From: "Murray, Jill" <JMurray@SantaBarbaraCA.gov>
To: <centralcoast@waterboards.ca.gov>
Date: 5/26/2009 3:10 PM
Subject: Basin Plan Triennial Review
Attachments: City of Santa Barbara Basin Plan Triennial Review.pdf

Please see attached comment form. <<City of Santa Barbara Basin Plan Triennial Review.pdf>>

> **********
> Jill Murray, Ph.D.
> Water Quality Project Coordinator
> Creeks Restoration / Water Quality Improvement Program
> City of Santa Barbara
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> Santa Barbara, CA 93102-1990
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CALIFORNIA REGIONAL WATER QUALITY CONTROL BOARD
Central Coast Region
895 Aerovista Place, Suite 101
San Luis Obispo, CA 93401

2009 BASIN PLAN TRIENNIAL REVIEW
COMMENT SUBMITTAL FORM

Please use this form to submit comments that you would like the Central Coast Regional Water Quality Control Board to consider during the Triennial Review of the Water Quality Control Plan for the Central Coastal Basin (Basin Plan). Comments submitted through the Triennial Review process provide the public with an opportunity to assist the Central Coast Regional Water Quality Control Board in identifying issues that need to be addressed through Basin Plan amendments in order to best meet the water quality planning needs of the Region.

The Central Coast Regional Water Quality Control Board is moving in a fundamentally new strategic direction, based upon a Vision of Healthy Functioning Watersheds. This new Vision represents a refocusing of our approach – a new framework for how we conduct business and achieve measurable results. The Vision structures our work towards our highest water quality priorities and more strategically aligns us with the anticipated challenges and opportunities in water quality and positions our agency to respond more nimbly to unexpected ones. For additional information about the Regional Board's Vision process, please see the following website: http://www.waterboards.ca.gov/centralcoast/publications_forms/publications/vision/index.shtml

Comments about any aspect of the Basin Plan are welcome. Of particular interest are comments about water quality standards (e.g., beneficial uses and water quality objectives) and comments that relate or align with the Regional Board's Vision.

Please email completed form(s) to centralcoast@waterboards.ca.gov. Please include the words "Basin Plan Triennial Review" in the subject line of your communication. Hardcopy forms may be mailed to the address in the header of this form, sent to the attention of Steven Saiz. Fax completed forms to (805) 543-0397. If you have multiple comments (i.e., multiple issues, concerns, suggestions), please submit a separate comment submittal form for each.

Deadline for submittal is Tuesday, **May 26, 2009 at 5:00 p.m.** Thank you for your participation!

1. CONTACT INFORMATION:

First Name:	Cameron
Last Name:	Benson
Organization Name:	City of Santa Barbara
Address:	620 Laguna Street
City:	Santa Barbara
County:	Santa Barbara
State:	CA
Zip:	93101
Telephone:	(805) 897-2658
Email:	cbenson@santabarbaraca.gov

2. DATE of COMMENT:

May 26, 2009

3. COMMENT:

a. Please specify the topic of your comment. (*Please try to limit topic to ten words or less*.)

Bacteria TMDL Approach Basin Plan Amendment

b. Please provide a detailed description of your issue/concern/suggestion, and explain why it needs to be addressed. (*There is no limit to the amount of text for the comment. The space will expand as needed*.)

The City of Santa Barbara requests that highest priority be given in the Triennial Review to the development of a Basin Plan Amendment outlining a rational, contemporary approach to Bacteria TMDLs, including TMDLs for beaches, in advance of beginning the Santa Barbara Beaches Bacteria TMDL process in earnest. Although a kickoff meeting was held in March 2008 and preliminary modeling was conducted, the project charter was never distributed, making this is an ideal time to pause and reflect prior to moving ahead with outdated objectives and approaches.

The current Bacteria TMDL approach outlined by the Board at the kickoff meeting uses standard indicators (fecal indicator bacteria) to assess water quality and develop implementation plans. The approach does not include mechanisms for focusing on the known limitations with indicator bacteria, namely that they can be derived from non-human or even non-waste sources, that they may not correlate with illness rates or more specific indicators such as enteric viruses, and that there is increasing evidence that they grow in the environment, e.g. on storm drains, sediment, or decaying kelp wrack (see attached). The impact of taking an approach that uses indicator bacteria concentrations exclusively is that implementation plans will focus on reducing indicator bacteria numbers first and foremost, rather than reducing the risk to human health. As a specific example, the City of Santa Barbara and UCSB have conducted testing for DNA-based human waste markers, and there has been no correlation between the methods. If we would have focused only on indicator bacteria hotspots, we may have put in diversions or other BMPs in the wrong places for reducing the risk to human health. In addition, some of the implementation tools suggested in other Bacteria TMDLs to reduce fecal indicator bacteria involve steps that are contradictory with the Board's Vision of Healthy Watersheds and goal of increasing healthy Aquatic Habitat, as described in the Brief Issue Descriptions for the Triennial Review process. For example, steps such as scaring birds away from beaches with loud noises or diverting water from stream channels could harm aquatic habitat. Prior to taking such steps, it should be confirmed that the source of indicator bacteria represents a true risk to human health.

The indicator bacteria method has been retained because it is cheap, easy to perform, and there has been nothing better available. However, after decades of little progress in addressing beach water quality nationwide, we are now at a very exciting time for the field, and the promise of routine use of indicators to correctly identify risks to human health is imminent. The USEPA is under consent decree to develop new recreational criteria that will be implemented by October 2012. In support of this objective, the agency has laid out a Critical Path Science Plan that outlines research to address several questions, including:

1. What is the risk to human health from swimming in water contaminated with human fecal matter as compared to swimming in water contaminated with non-human fecal matter?
2. How well do culture and molecular methods for various indicators (singly or in combination) correlate with swimming-related illnesses?
3. Are the indicators, methods and models suitable for use in different types of waters and for different CWA [Clean Water Act] programs?

In addition, regulatory developments have occurred since the Santa Barbara Beaches TMDL kickoff that illustrate the need for an Amendment. As background information, note that based on material provided for stakeholder and kick off meetings for the Santa Barbara Beaches TMDL, it was suggested that a reference beach approach would likely be used, following the lead of Los Angeles and San Diego. While aspects of the reference beach approach are desirable, and it should be included in the Amendment, there are four major problems that suggest it should not be the default or exclusive approach used. First, as outlined above, the approach takes a blanket approach to reducing indicator bacteria concentrations, without focusing on those posing the greatest risk. Second, there are very few reference beaches available (e.g. the San Diego TMDL uses Leo Carrillo in Los Angeles County as the reference beach). There should be multiple reference beaches for varying beach types, i.e. sheltered, kelp-laden, cobble-strewn, etc. Third, the choice of statistics for choosing the time period for the data set is arbitrary and may not be protective enough for human health. Fourth, by requiring that a reference beach be in an undeveloped watershed, it effectively deems all increases in fecal indicator bacteria over the reference beach as harmful, and does not allow that increased concentrations of harmless indicators may be related to numbers of storm drains and impervious surfaces, rather than fecal input. This may also discourage the implementation of restoration projects that may lead to increased birds and wildlife, due to potential fecal inputs.

The San Diego Beaches TMDL moved forward with the reference approach despite a lack of appropriate reference beach(es) and a request by stakeholders to use the natural exclusion approach. The TMDL notes that the natural exclusion approach will be addressed in the first review. The State Water Board recently approved on consent an Amendment to the San Diego Basin Plan that directly outlines bacteria objectives and allowable TMDL approaches, including the reference beach and natural exclusion approaches. The natural exclusion approach directs the reduction of anthropogenic indicator bacteria (typically from human and domestic animal waste) first, and allows exceedances based on “natural, uncontrollable sources.” The Amendment states explicitly, “It is not the intent of the [San Diego] Regional Board to require treatment or diversion of natural water bodies or to require treatment of natural sources of bacteria. Such requirements, if imposed by the Regional Board, could adversely affect valuable aquatic life and wildlife beneficial uses supported by water bodies in the Region.” Now that the Amendment has received State Water Board approval, the San Diego TMDL will likely be revised, involving numerous hours of staff time and stakeholder input.

The City believes that the Central Coast Regional Board would save the community significant resources and lead to a higher chance of protecting human health if it addresses the larger picture approaches and goals of the TMDL prior to proceeding with writing the Project Charter and Project Report. The City urges the Board to take into consideration the rapid advancement of science in this field and either postpone the Beaches TMDL until the

epidemiology studies, indicator development, and new criteria have been released or develop an Amendment that allows flexibility as new data are generated.

The City recommends that the Basin Plan Amendment contain the following sections, drawing heavily from the San Diego Basin Plan Amendment, "A Resolution Amending the Water Quality Control Plan for the San Diego Basin (9) to Incorporate Implementation Provisions for Indicator Bacteria Water Quality Objectives to Account for Loading from Natural Uncontrollable Sources Within the Context of a Total Maximum Daily Load:"

1. Updated REC-1 objectives.
2. A recommendation for immediate reconsideration of the REC-1 objectives upon the USEPA's release of new criteria in 2012.
3. A statement confirming that the driving motive is to protect human health during recreational contact.
4. Prioritization of reducing indicators/sources based on their likely impact on human health: first, human waste/sewage; second, domesticated animal waste, and third, wild animal waste.
5. A statement confirming that reduction of indicators that do not relate to human health risk, e.g., indicator bacteria growing in the environment is not a goal of the TMDL.
6. Approved approaches to the TMDL, including a reference beach approach and a natural exclusion approach. For both approaches, it should be noted that urbanization, i.e. undergrounding of natural stream channels, leads to increased indicator bacteria that are not necessarily indicative of human or animal waste.
7. A review of the SHELL beneficial use designation on the Central Coast.

The City feels that the amendment proposed here meets the proposed ranking criteria as follows:

1. Vision Alignment: The issue is aligned with the Vision of Healthy Watersheds and Measurable Goal of protecting Aquatic Habitat because it seeks to find solutions to recreational exceedances that have the greatest ability to protect human health while minimizing unnecessary harm to the aquatic habitat.
2. Water Quality Standards Improvement. The proposed amendment will improve water quality standards by bringing them in line with the state-of-the-science and with recent regulatory changes, along with providing room for adapting objectives based on advances in research and the EPA's upcoming revised criteria.
3. Effectiveness. The proposed amendment will improve clarity and consistency to the TMDL process, and improve coordination among staff and programs at the Board that involve monitoring, listing water bodies, revising beneficial uses, and developing implementation plans.
4. Public Interest. There is high perceived public interest in the Santa Barbara Beaches TMDL, as demonstrated by the high turnout at the TMDL kickoff meeting.

4. GEOGRAPHIC SCOPE OF COMMENT:

Mark the box ( ) that best corresponds to the geographic scope of your comment:

☒	Entire Central Coast Region
☐	Multiple watersheds

	If the watersheds and/or waterbodies are known, please specify here:
☐	Single watershed
☐	Multiple waterbodies
☐	Single waterbody
☐	Beach or coastal waters
☐	Other:
☐	None of the above (comment is administrative or has no direct geographic scope)

5. ADDITIONAL INFORMATION ABOUT YOUR COMMENT:
Information about the following items may help us better understand and evaluate your comment. For any that are applicable to your comment, please elaborate in the space provided. Leave blank if item is not applicable or if you are unsure.

a. If you think a Basin Plan amendment addressing your comment would likely have widespread stakeholder support, please explain in the space below and, if known, list supportive stakeholder(s) with phone or email contact(s). (*The space will expand as needed.)*

The City of Santa Barbara can provide contacts upon request.

b. If substantial resources have been invested in developing technical information that would support a Basin Plan amendment addressing your comment, please explain in the space below. (*The space will expand as needed*.)

The City of Santa Barbara has spent over $350,000 of Measure B funds in developing, testing DNA-based microbial source tracking tools to correctly identify types and locations of sources of waste to drains, creeks, and beaches. In addition, the City has received over $400,000 in grant funding from the State Water Board's Clean Beaches Initiative Program to continue developing and testing these tools. Finally, the substantial resources are being spent by other agencies and researchers, as displayed at the recent USEPA Beaches Conference, in efforts to improve testing methods, source tracking, and TMDL processes (see reference section).

c. If substantial resources are likely available to augment Regional Board resources needed to develop a Basin Plan amendment addressing your comment, please explain in the space below. (*The space will expand as needed*.)

The San Diego Basin Plan cited above can serve as a template for the beginning the process, saving substantial staff effort in developing an amendment for the Central Coast.

6. HOW TO BRING ADDITIONAL INFORMATION TO OUR ATTENTION:

a. If you would like to direct us to additional information that supports/supplements your comment, e.g., web address, report citation, contact person for follow-up, please give direction in the space below. (*The space will expand as needed*.)

San Diego Basin Plan Amendment: A Resolution Amending the Water Quality Control Plan for the San Diego Basin (9) to Incorporate Implementation Provisions for Indicator Bacteria Water Quality Objectives to Account for Loading from Natural Uncontrollable Sources Within the Context of a Total Maximum Daily Load.

http://www.waterboards.ca.gov/sandiego/board_decisions/adopted_orders/2008/R9-2008-0028.pdf

Technical Report supporting Basin Plan Amendment above:
http://www.waterboards.ca.gov/sandiego/water_issues/programs/basin_plan/docs/amendments/issue_7/7-25-08update/Final_Technical_Report_June08.pdf

USEPA Presentation on New Recreational Criteria given at the 2009 Beaches Conference:
http://epa.gov/waterscience/beaches/meetings/2009/pdf/beach_session_tue_break.pdf

For additional information, see USEPA Technical Document on Criteria Development:
http://www.epa.gov/waterscience/criteria/recreation/plan/developmentPlan.pdf

USEPA Critical Path Science Plan, outlining ongoing state-of-the-science and ongoing studies:
http://www.epa.gov/waterscience/criteria/recreation/plan/cpsplan.pdf

City of Santa Barbara Microbial Source Tracking Report, demonstrating how non-indicator bacteria tecniques are used to identify human waste:
Laguna Watershed and Water Quality Improvement Feasibility Analsyis
http://www.santabarbaraca.gov/NR/rdonlyres/B6A274E7-3D46-4E51-B7F8-C04334477829/0/LagunaWatershedStudyFINAL.pdf

b. If you are submitting additional information that supports/supplements your comment, e.g., reports, articles, data sets, please tell us what you are sending so that once received we can link it to your comment. (*The space will expand as needed*.)

We are submitting electronically a second microbial source tracking report, the Final Report produced by UCSB, outlining methods development, testing, and use of DNA-based methods to identify locations and sources of contamination in storm drains.

We are also submitting an informal report by Santa Barbara County illustrating rapid growth of indicator bacteria on kelp.

Note: Supporting information may be emailed to centralcoast@waterboards.ca.gov; sent to the attention of Steven Saiz at the address in the header of this form; or faxed to (805) 543-0397. Please include the words “Triennial Review” in the subject line of your communication.

Our email system can accommodate files up to approximately 15MB. If you are uncertain of how best to submit additional supporting information, please call 805-549-3879.

Thank you for participating in the Triennial Review of the Basin Plan!

For additional information about the Triennial Review process, please see the following website:
http://www.waterboards.ca.gov/centralcoast/publications_forms/publications/basin_plan/triennial_review/index.shtml

From: "Murray, Jill" <JMurray@SantaBarbaraCA.gov>
To: <centralcoast@waterboards.ca.gov>
Date: 5/27/2009 10:23 AM
Subject: Triennial Review
Attachments: City of SB Kelp Regrowth Study.pdf; City Final Report_5 with Draft wm.pdf

Hi there,
I realized I forgot to send two attachments yesterday to the letter for the City of Santa Barbara <<City of SB Kelp Regrowth Study.pdf>> <<City Final Report_5 with Draft wm.pdf>> .
Thanks,
Jill

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DNA-Based Source Tracking of Human Fecal Material in Santa Barbara, California:

Report of a Research Project for the City of Santa Barbara

Submitted on behalf of the University of California by

Patricia A. Holden, Ph.D., P.E.
Principle Investigator
Bren School of Environmental Science & Management
and the Marine Science Institute
University of California, Santa Barbara, CA 93106-5131

August 30, 2007

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Acknowledgements

The research described in this report was funded by the City of Santa Barbara through Measure B funding, and by the Switzer Foundation through a Leadership Grant to the City.

The research was substantially performed, including the writing of this report, by Laurie C. Van De Werfhorst and Dr. Bram Sercu in the Holden Lab Group at UCSB. Others in the Holden Lab who contributed to field and laboratory research included Scott Olson, Dr. Noah Fierer (visiting postdoctoral researcher), George Weber, Rachel Steinberger, Erin Nuccio, and Allison Horst.

This research was performed cooperatively with the City through the assistance of Dr. Jill Murray, Jill Zachary, Tim Burgess, Harry Slicker, Steve Mack, Rebecca Bjork, additional staff of the Creeks Division, and staff of the El Estero Wastewater Treatment Plant.

We thank Nancy Callahan of the Santa Barbara Wildlife Care Network for making her facility available for the source of raccoon scat.

Weekly ocean water quality data from the Santa Barbara County, Department of Environmental Health Services, was co-evaluated in this report alongside data collected specifically for this study. We acknowledge the assistance of Willie Brummett and Robert Almy with Santa Barbara County.

Flow data for Mission Creek and Arroyo Burro Creek was provided through the NSF-funded Santa Barbara Long Term Ecological Research project. We acknowledge the assistance from Scott Coombs and John Melack.

The rhodamine tracer study in Mission Creek was planned and performed collaboratively with Professor Jordan Clark of UCSB and his students, William (Billy) E. Szafranski, and Scott P. Walls.

We thank MarBorg Industries for assistance in acquiring the samples of septage and septic tank solids used in this study.

We thank the Creeks Restoration and Water Quality Improvement Citizens Advisory Committee for providing valuable feedback at interim review periods during the course of this project.

Microarray (PhyloChip) analysis of DNA from the Mission Creek Phase II 'snapshot" study was performed in the lab of Dr. Gary Andersen of Lawrence Berkeley National Laboratory. Contributors to this work included Dr. Cindy Wu and Dr. Todd DeSantis. Also acknowledged are Dr. Terry Hazen and Dr. Eoin Brodie for their contributions to this portion of the study.

Executive summary

Background

UCSB was contracted by the City of Santa Barbara (City) to perform research in support of the project "DNA – Based Source Tracking of Human Fecal Material", as described in a proposal with the same title. The contracted project period was originally June 15, 2004 – December 31, 2005, and was extended twice at no additional cost to the City, ultimately to August 31, 2007. The initial extension, to June 30, 2007, allowed for additional field and laboratory work, plus associated data analysis, beyond the original scope. The 2nd extension allowed for final delivery of data to UCSB from a vendor, Lawrence Berkeley National Laboratory, for services associated with one of the added scope items. Due to administrative delays in finalizing the contract between UCSB and the City, the actual project work officially commenced on August 10, 2004 when the first official working meeting was held by UCSB and the Creeks Division.

Project Objectives

The overall objective of the research was to determine the possible presence and potential origins of human waste in lower Arroyo Burro and Mission Creeks at sites including several inland as well as in associated coastal lagoons, and in the surf zone at associated beaches. The specific geographical focus of this study was originally planned for three areas: 1) old Mission Creek from Bohnett Park into the new Mission Creek concretized channel, 2) mid and lower Mission Creek extending to the ocean and including the lagoon, and 3) lower Arroyo Burro Creek with an emphasis on lagoon and beach processes. A two phase approach was planned: Phase I was to determine the efficacy of two DNA-based tests for discerning human from animal fecal material in environmental matrices. Phase II was to apply the demonstrated assays to understanding the origins and fates of human fecal material in the study areas. This project was to be conducted cooperatively with the City of Santa Barbara Creeks Division in that the detailed sampling locations and objectives for various sub-studies conducted in Phase II were planned in consultation with Creeks Division staff.

Project Scope of Work

The scope of work performed was substantially responsive to the original project objectives. During the course of the research, subprojects not originally delineated in the contract were nominated by UCSB and approved by the City. All tasks (and time frames), i.e. those originally contracted and those that were added or modified from the original scope, are listed below:

- Initial work planning (August 2004): choose host animals and sampling sites for Phase I
- Phase I (September 2004 – May 2005): test and evaluate selected (DNA-based and traditional indicator organism-based) methods for assessing water quality and for discerning fecal material from background matrices of water, sediments, and soil.
- Phase II (June 2005 – May 2006): use the DNA- and traditional indicator-based methods from Phase I to characterize water quality at locations of interest to the City, extending from lower Mission Creek and Arroyo Burro Creek to the coastal ocean. The two DNA-based methods evaluated during Phase I (routine polymerase chain reaction or PCR of the gene marker for human waste-associated *Bacteroides*) were further refined, including adopting a newer version of the *Bacteroides* method, i.e. a quantitative PCR or qPCR method, to determine quantities of gene markers instead of only their presence or absence. Phase II included six subprojects:
    - IIA: a 3-sequential-day "snapshot" study of lower Mission Creek,
    - IIB: source-tracking at Haley Drain plus a fate and transport study downstream of Haley Drain,
    - IIC: a 3-sequential-day "snapshot" study of Arroyo Burro Creek,
    - Clone Library: DNA-based analysis of cultivated fecal indicator bacteria (FIB) to determine which organisms grow in standard FIB assays,

- Storm: determine DNA- and indicator organism-based microbiological water quality in Arroyo Burro Creek and Mission Creek during two "New Year" storms in late December, 2005 and early January, 2006,
- Dispersion: perform a dye study in the Mission Creek reach between Haley and Gutierrez Streets to characterize the degree of longitudinal mixing in the water as it flows from up to downstream. This provided a parameter value relevant to modeling the fate of introduced contaminants in Creek water.

- Phase III (June 2006 – August 2007): use methods (as in Phase II) to further characterize microbiological water quality and possible origins of human waste in the storm drain systems upstream of Hope and Haley Drain discharges. Additionally, further analyze DNA extracted from selected Phase IIA samples for the presence of pathogen groups using a new technology developed by Lawrence Berkeley National Laboratory (LBNL) called the "PhyloChip". The subprojects were:
  - Haley Drain Tracking: sample within the Haley Storm Drain system to determine possible origins of human waste,
  - Haley Sediments: sample sediments in Mission Creek, downstream of Haley Drain discharge, to determine potential storage of human waste markers in sediment matrices,
  - Hope Drain Tracking: sample within the Hope Storm Drain system to determine possible origins of human waste,
  - Carrillo and Victoria Drain sampling: sample the discharge of each drain in a "snapshot" study to determine if human waste markers are present,
  - PhyloChip analysis: submit DNA to LBNL for PhyloChip analysis, and cooperatively analyze data,
  - Historical FIB Analysis: analyze historical FIB data provided by the City to assess relative trends between sites studied in this research on Arroyo Burro and Mission Creeks.
- Progress reporting: provide periodic written progress reports to the City, and present interim progress twice (2005 and 2006) to meetings of the Citizens

Advisory Committee (CAC) for Creeks Restoration and Water Quality Improvement. Present preliminary data to scientific conferences (in this case to the annual General Meeting of the American Society for Microbiology in 2006 and 2007).

Organization of this Report

This report is organized into the following chapters that convey the work performed and results of the associated studies:

- Chapter 1: Analysis of Two DNA-Based Approaches for Fecal Source Tracking (Phase I research)
- Chapter 2: Microbiological Water Quality and Fecal Source Tracking in Two California Coastal Creeks (Phase IIA, Phase IIC, and the Phase III Haley Drain Tracking and Hope Drain Tracking studies)
- Chapter 3: Fate and Transport of Human Waste Downstream of a Storm Drain Discharge (Phase IIB and the Phase II Dispersion study, plus the Phase III Haley Sediment study)
- Chapter 4: Microbiological Quality of Storm Flow in Two California Coastal Creeks (Phase II Storm study)
- Chapter 5: Source-Water Dependent Growth of Non-Target Bacteria in Colilert and Enterolert Fecal Indicator Assays (Phase II Clone Library analysis)
- Chapter 6: High Density Microarray Analysis of Water Quality in a California Coastal Creek (Phase III PhlyoChip analysis)
- Chapter 7: Microbiological Water Quality of Carrillo and Victoria Drains Discharge (Phase III Carrillo and Victoria Drain Sampling)
- Chapter 8: Historical Analysis of City FIB Data for Lower Arroyo Burro and Mission Creeks
- Appendices (Digital only): As per the Contract, data are provided in a form that can be used by others. Because of the volume of data generated, it is not provided in print form.

Chapters 1 and 2 are based on materials delivered to the City in progress reports in June, 2005 and July, 2006, respectively. Additional work has been performed since the

2006 report and thus Chapter 2 is a more current reflection of the entire related work. Chapters 3 through 8 contain material that has mostly not been presented to the City in the form of a written report. Chapter 5 has been submitted for publication to a scientific journal. Chapters 2 and 4 have been similarly formatted for submission to a scientific journal in the near future. Chapters 1 through 8 describe the methods, results, discussion, and references to published literature. As per the Contract, data associated with this research are provided to the City in digital form.

Summary of Findings: DNA-Based Source Tracking of Human Fecal Material

The DNA-based methods used in this study included routine PCR and qPCR of a gene marker for a human waste-associated strain of *Bacteroides*, hereafter termed the human gene marker or HGM, and analysis of whole bacterial communities by terminal restriction fragment length polymorphism (TRFLP) analysis. As expected, the HGM was only detected in waste containing human fecal material (i.e. sewage, septage and raw human fecal material); DNA from the feces of other animal hosts including raccoon, seagull, dog and cat, did not contain the HGM. These results confirm the specificity of the HGM method to human waste for the purposes of this study. Similarly, TRFLP profiles from DNA isolated from the various waste sources were reasonably distinct, implying that this method would be useful for discerning the relative influence of various wastes on water quality. Because host-specific gene markers were not available for gull, raccoon, and dog at the time of this study, the community profiling, or similar, method was needed to relate the DNA of environmental waters to prospective host profiles. Sewage spiked into environmental waters (creek and ocean water) was indicated by TRFLP profiles and detected by analysis for the HGM. Similarly, human waste was indicated by TRFLP when spiked into creek water in the laboratory. Due to either insufficient recovery of DNA or organic chemical inhibitors (e.g. humic substances) to PCR, the attempt to apply these two DNA-based methods to fecal-spiked sediments and soils was not successful. FIB concentrations in sewage and septage were within reasonable expected ranges. Based on the overall Phase I results, both TRFLP and HGM

DNA-based approaches were applied for the rest of this research which was more focused on water rather than on either sediments or soils.

Snapshot sampling (3 successive days, multiple sites) of lower Mission and Arroyo Burro Creeks, the coastal ocean at Hendry's and East Beaches, Mission and Arroyo Burro Lagoons, and Haley and Hope Drains, during dry weather in the summer of 2005 was used to survey for human fecal material and the possible influences of other fecal sources on microbiological water quality. Sites for sampling were selected with City consultation. The snapshot studies strongly supported that the two storm drains, i.e. Hope and Haley Drains, were discharging human waste as shown by their relatively high HGM content. The HGM was quantifiable at other sites but not on all days, and at lower levels. Overall, spatial and temporal variability in microbiological water quality was high among the sites. A fate and transport study downstream of Haley Drain further indicated that the HGM concentrations are highly variable in space and time, perhaps due to diurnal variations in flow. The dispersion coefficient for the reach between Haley Street and Gutierrez was extremely low, implying minimal longitudinal mixing and thus the probable importance of decay in microbial signals from up to downstream. The HGM was detected upstream of Haley Drain in the storm drain system, indicating a distant source. Yet television of the storm drain (by the City) between the Haley Drain discharge and the intersection of Chapala and Haley Streets did not reveal any obvious cross-connections. Taken together, both creek reaches were contaminated with human-associated waste and evidence of such was periodically found in the lagoons. Preliminary estimations of possible transport characteristics of such contamination suggest that there are conditions that could allow migration of upstream creek contamination to the coastal ocean, thereby contributing to unhealthful waters. Consistently, the HGM was quantified in ocean water at Hendry's Beach on one out of three sampling days.

In response to questions raised during the 2006 progress presentation to the CAC, scientists at LNBL were engaged to further analyze Mission Creek DNA samples associated with the 2005 snapshot study. The lab of Dr. Gary Anderson (LBNL) has

created a microarray (gene chip) technology called the "PhyloChip" that contains the most comprehensive library of microbial gene sequences available. Marriage (termed hybridization) of sample DNA to the PhyloChip was intended for further understanding the possible presence of pathogens in creek water. While the summer 2005 DNA samples clearly showed the presence of human waste in both creeks, PhyloChip analysis could more definitively determine if microbial pathogen groups were in the water. Some pathogen groups were shown to be absent (e.g. *Campylobacter jejuni*), although there was evidence for the occurrence of other pathogen groups throughout lower Mission Creek (e.g. *Helicobacter pylori*). PhyloChip technology cannot indicate actual infectiousness of detected pathogens, nor is it absolutely quantitative, however its use here afforded a higher resolution analysis of all known microbes and thus pathogens. The PhyloChip confirmed that some samples having detectable HGM concentrations were related to human fecal waste, although it also indicated the presence of traces of human fecal waste in Old Mission Creek, a location where HGM had not been detected with qPCR.

In the summer of 2006, the City began diverting Haley and Hope Drain discharges away from Mission and Arroyo Burro Creeks and into the sanitary sewer during the dry weather months. Therefore, research in the summer of 2006 was not focused on creek waters downstream of the drains, but was focused on "drain tracking" to determine potential origins of human waste in the two storm drain systems. Research was also towards sampling additional drain discharges. Further research of the Haley Drain storm drain system in the summer of 2006 yielded FIB and HGM data consistent with an upstream source. The absolute source was not pinpointed, but HGM and FIB concentration patterns suggest that a possible future focus should be at the Haley and Chapala intersection. Similarly, the drain system upstream of the Hope Drain discharge was sampled for HGM and FIB. However, HGMs were only consistently detected in one of several successive sampling days and only at two points upstream: first in a catch basin on State Street near the intersection with La Cumbre Road, and second in the Hope Diversion structure during a transient period of increased flow. There was also evidence of HGM at one drop inlet and an additional manhole in the La Cumbre storm drain

system, but levels were within analytical variation of the qPCR method for HGM. Both drain tracking studies suggest strongly that human waste-associated water contamination is highly variable in time, possibly due to periodic dilution effects of transiently high flows. Additional research is needed to test this hypothesis and to apply the principle of measuring waste "load" (the product of water flow and waste marker concentration) to discovering the exact spatial origin of contamination. Also, additional research would be required to determine if more storm drains in the City are discharging HGM and associated waste to water bodies. During the summer of 2006, Carrillo and Victoria Drain discharges were sampled on 3 successive days, but the HGM marker was detected only once in each drain in replicate analyses.

The taxonomic groups of bacteria in FIB assays, by clone library analysis, varied with sample type. Specifically, the FIB that grew in water samples acquired during a storm in early November, 2005 were different than FIB grown from dry weather flow sampled a few weeks prior. This result raises questions about the use of FIB analyses in winter storm conditions when erosion of soils can contribute many non-target organisms to the assays. Consistently, during the New Year's events in late 2005 / early 2006 when storm flow was high, microbial communities (by TRFLP profiling) in creek water appeared distinct from those found in during dry weather. FIB concentrations were high during the storm, but not necessarily higher than dry weather. Also, HGMs were not detected in storm flow samples. These results suggest that either human waste sources are diluted or more attenuation of bacteria associated with those sources occurs in storm flow as opposed to dry weather flow. Further, the clone library study and storm study support that FIB in creek water arise from more different sources during wet weather than in dry weather.

This research provides a much better understanding of microbiological water quality in lower Arroyo Burro and Mission Creeks, including the possible relationships of water quality to urban infrastructure such as storm drains. This study also demonstrates the efficacy and utility of using the DNA-based methods researched here. Clearly, dry weather water quality in lower Arroyo Burro and Mission Creeks was impacted by

human waste sources, and the identified sources were found to be associated with storm drain infrastructure. Thus, this study successfully tracked human sources of creek water contamination to urban storm drains. However, exactly where or how human waste entered storm drains was not determined in this study. Subsequent research should focus on systematically quantifying the origin of contamination in storm drains such as the Haley Drain. This would likely involve synoptically measuring water flow when samples are acquired, since tracking waste on only the basis of HGM or other marker concentrations can be confounded under transient flow conditions. Additional research is also needed to more completely determine the extent of human waste discharges across other drains in the City. Also, additional research is needed to understand the relationships between upstream human waste contamination in creeks and downstream beach microbiological water quality. This study suggests the potential for creeks to deliver human waste-associated microbes from upstream creek sites to the coastal ocean; this study also detected human waste in the coastal ocean. However, the study results also support that the coastal ocean has a distinct microbial community which could also indicate that other sources of FIB, potentially in the local beach environment, may be contributing to poor microbiological water quality at Hendry's and East Beaches. Additional research would be necessary to discover the relative importance of local (beach-related) sources versus upstream (creek-related) sources in contributing to poor water quality at these beaches.

Chapter 1: Analysis of Two DNA-Based Approaches for Fecal Source Tracking (Phase I research)

1.1 Introduction

The purpose of the Phase I sampling and subsequent research was to test DNA-based and traditional (indicator organism-based) methods for assessing water quality and for discerning fecal material from background matrices of water, soil and sediments. Following preliminary data collection and methods optimization during Fall 2004, two sampling events in support of Phase I research were conducted: December 14, 2004 for Mission Creek and December 17, 2004 for Arroyo Burro. Key results were:

1) DNA-based methods were suitably sensitive for distinguishing the presence of sewage in creek waters
2) Septic solids and sewage appeared distinct by the DNA-based methods used
3) Bohnett Park Creek water did not show evidence of human fecal or sewage contamination by either DNA-based method despite high indicator organism concentrations
4) Bacterial DNA from ocean water at both Mission and Arroyo Burro Creek outlets was distinct from creek water and any of the contamination sources.

1.2 Materials and Methods

1.2.1 Sites and Sampling

Two watersheds, Mission Creek and Arroyo Burro, in the City of Santa Barbara, CA were targeted for study. For each, an upstream, relatively pristine, reference site and downstream, relatively urbanized, study sites were selected for sampling creek water, sediments and bank soils. Reference sites were chosen because of their waters' consistently (from multi-year, monthly sampling) low fecal indicator bacteria concentrations. Study sites were chosen because waters there frequently had contained high indicator bacteria concentrations. The reference site for the Mission Creek watershed was located on a tributary, Rattlesnake Canyon Creek, just upstream of a

bridge crossing at Las Canoas Road. The study sites for the Mission Creek watershed included one at Old Mission Creek, just downstream of Westside Drain stormwater discharge which flows through a public park and playground (Bohnett Park), and another in the surf zone where the Mission Lagoon discharges into the Santa Barbara Channel. For the Arroyo Burro watershed, the reference site was just upstream of the discharge of Veronica Springs into Arroyo Burro Creek; the study sites included Arroyo Burro Creek just upstream of Arroyo Burro Lagoon, the Lagoon itself, and the surf zone just downstream of the Lagoon discharge into the Santa Barbara Channel.

The two watersheds were sampled three days apart with Mission Creek on December 14, 2004 and Arroyo Burro on December 17, 2004. While at least two major rain events had occurred earlier in the season, less than ½" had fallen in the previous 2 weeks and no rain had fallen within 6 days prior to the first day of sampling. All sampling took place in the morning so that sample processing could be accomplished well within 1 day. Grab samples of water were taken with a sterile beaker and composited at the time of sampling into 20 liter sterile polypropylene carboys. Sediments and unvegetated creek bank soils were scooped using Samplit™ Sterile Scoop & Container Systems (Sterileware®), which consisted of sterile containers with scoops integral to the caps.

Fecal source material was sampled on the same days and time frames as associated watersheds. The fecal sources sampled in conjunction with Mission Creek watershed included humans, seagulls, sewage from the influent of the El Estero Wastewater Treatment Plant (WWTP, City of Santa Barbara, CA), and raccoons as these were deemed, through consultation with the City, key suspected contributors to fecal material in creek and coastal waters. Human feces from 3 individuals were acquired from a local hospital lab. Gull feces were collected by baiting onto clean plastic tarps. Feces from a minimum of 3 birds were scraped with a sterile Samplit™ Scoop and composited into the attached sterile vessel on site. Raccoon feces from 3 healthy animals were similarly scooped and composited from individual cages. Raw sewage was acquired mid-morning from the El Estero WWTP. The sources sampled in conjunction with Arroyo Burro Creek watershed included septic solids, dogs and cats. Septic solids, representing the composite liquid material from several residential tanks, was acquired

from a local pumping company (MarBorg, Santa Barbara). Dog feces were acquired from three healthy individuals (male Sheppard mix, female Retriever, female miniature Dachshund), each from a separate household. Cat feces were acquired from three healthy individuals (all male, mixed breeds) of two separate households.

Watershed and fecal source samples were maintained on wet ice (4º C) during the sampling period and during sample processing. Sample processing and analysis began well within 6 hours of sample collection.

1.2.2. Fecal Spiking of Environmental Matrices

The primary uses of water, soil and sediment samples from reference sites were as matrices into which various combinations and proportions of fecal sources were spiked and subsequently analyzed to determine if added fecal sources could be resolved against the natural background. Unspiked reference site samples served as controls. Samples from urbanized, downstream study sites were not spiked with fecal materials; these samples were regarded as unknowns for which the presence of fecal bacterial DNA would be determined.

Water samples were mixed well by shaking (5 sec) before each aliquot was removed for matrix spike preparation. Soils were sieved to 2 mm, and sediments and soils were homogenized thoroughly by stirring prior to use as matrices for fecal spikes.

Solid feces (from gull, raccoon, dog, cat and human) were thoroughly composited for each host animal before 0.25 g of composited feces were archived (-20º C) for later DNA extraction and 1 g was weighed for oven dry (105º C) solids determination (3). For the purposes of spiking feces into matrices, a ca. 2% (by weight) solution was prepared by adding a composite feces into reference creek water. Sewage and septic solids samples were shaken thoroughly (5 sec) prior to subsampling. Ten mL of sewage and septic solids solution were individually centrifuged (10,000 g, 10 min.) to recover solids for archiving (-20º C) and later DNA extraction. Preliminary analysis (TRFLP) of PCR-amplified genes encoding bacterial community 16S rRNA from centrifuged raw sewage (El Estero WWTP), as well as from bulk sewage, indicated that the pellet, supernatant and bulk communities were identical (data not shown). For the purposes of spiking

sewage into matrices, a 1% (by volume) diluted stock was prepared in reference (Rattlesnake Canyon of Mission Creek watershed) creek water. Raw septic solids solution and sewage contained different solids contents which were approximated by centrifugation (10,000 g, 10 min.) of equal volumes (30 mL) and weighing the pellets. Based on this method, raw septic solids solution was pre-diluted into sterile Nanopure water to approximately match the solids content of the raw sewage. A 1% septic solids stock was prepared in reference (Veronica Springs of Arroyo Burro watershed) creek water from the diluted septic solids solution.

Sewage was spiked into Rattlesnake Canyon reference water at four percentages with the lowest selected to approximately simulate the total coliform concentration that would minimally meet the California recreational ocean water quality standard. Various combinations of fecal sources were selected for spiking based on anticipated co-occurrence in the field and at levels thought to be realistic. For the purposes of planning dilutions and spike proportions, the following assumptions were made: 1) raw sewage total suspended solids (TSS) and total coliform concentrations were 1000 mg/L and 10^6 / mL, respectively (5), 2) solid fecal sources were 50% water, 3) septic solids TSS concentration was 50,000 mg/L, 4) total coliform concentration in animal feces including human was 10^7/g, 5) total bacteria concentration in soil was 10^6 / g, and 6) total bacteria concentration in sewage was 10^8/mL. Recipes used in preparing the mixtures are listed in Tables 1-1 through – 1-4.

1.2.3 Indicator Organism Enumeration

Based on the assumptions (above) regarding solids concentration and indicator organism concentrations in fecal sources, spiked water and soil or sediment slurry samples were diluted in sterile water prior to indicator organism enumeration by the defined substrate methods (IDEXX Corp.). Where applicable, i.e. for creek, lagoon and ocean water samples, IDEXX data from this study were compared to data from the same sites recently sampled by the City Creeks Division.

1.2.4 DNA Extraction, Quantification, and PCR Amplification

Total DNA was recovered using the UltraClean™ Water DNA Kit (MO BIO Laboratories) for approximately 1-2 L of each liquid sample which included reference waters, study site water samples and reference waters spiked with fecal materials. For these samples, further concentration was by ethanol precipitation. For soil and sediment slurries spiked with feces, solids were separated from 100 mL by centrifugation (10,000 g, 10 min) and the pellet was archived (-20º C). DNA was recovered using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories) for solid samples (0.25-1 g wet weight) which included unspiked soil and sediments, solid fecal materials, and pellets from centrifuged sewage, septic solids and spiked soils and sediments. The purity of the DNA extracted from sewage was previously determined to be sufficiently high for PCR amplification (data not shown). Total DNA was quantified with the Quant-iT™ PicoGreen® dsDNA kit (Molecular Probes/Invitrogen) using either calf thymus DNA or the supplied lambda DNA as the standard.

DNA was PCR amplified using standard methods (4) and using primers for *Bacteriodes-Prevotella* group genetic markers specific to humans (1, 2). PCR products from the amplification of human specific *Bacteriodes-Prevotella* were resolved by agarose gel electrophoresis; a positive sample was scored if there was a visible band the same size as in the positive control (sewage).

1.2.5 Bacterial Community Analysis by TRFLP

Restriction and sequencing of terminal restriction fragments was performed as before (4), except that the PCR products were purified using QIAquick PCR Purification Kits (QIAGEN) and were only restricted with *Hha*-I. Peaks were normalized as Dunbar et al. (6), aligned using a binning method, and analyzed after log or presence/absence transformation. Statistical analyses of TRFLP profile similarities were performed by Principle Components Analysis (PCA) in the software program PC-ORD (version 4, MJM Software).

1.3 Results

1.3.1 IDEXX

Results of the IDEXX assays are provided in Tables 1-5 and 1-6. As shown in Tables 1-5 and 1-6, some samples were insufficiently diluted to provide indicator organism concentration data. Other samples were over diluted. Many dilutions resulted in useable estimations of indicator organism concentrations.

To determine consistency between these results and those recently generated for select sites by the City Creeks Division and the County of Santa Barbara Environmental Health Services Department, bar charts were generated for comparison (Fig. 1-1 through 1-7). Based on these comparisons, most of which represent a temporal sequence, the FIB data resulting from this study appear reasonably within ranges observed by the City.

1.3.2 Bacteroides

The results from PCR amplifying human waste markers based on *Bacteroides-Prevotella* group primers were such that sewage and all sewage-spiked waters were positive for the HGM. There were no false positives detected in the spiked samples. Insufficient DNA was recovered from the Rattlesnake Canyon water sample that was spiked with 0.01% sewage, the Rattlesnake Canyon water by itself, the Rattlesnake Canyon sediment samples that were spiked with sewage alone or with sewage/gull/raccoon, the Veronica Springs sediment sample that was spiked with septic solids, and the Veronica Springs water sample that was spiked with 0.1% septic solids. Human and human spiked samples, septic solids, Veronica Springs water spiked with 5% and 1% septic solids, with high septic solids/cat/dog, with septic solids/cat, and with septic solids/dog all generated a "partial" positive signal. A band of the correct size was present on the gel, but it was fainter than the positive control (sewage). The remaining septic solids spiked waters, sediments and soils did not generate a positive HGM signal. It should be noted that this partial positive/negative result from the septic solids, which differs from sewage, is also supported by the TRFLP data that clearly distinguishes

sewage from septic solids. None of the unspiked environmental (study) waters, soils or sediments were positive for the HGM.

1.3.3 TRFLP

TRFLP electropherograms suggested differences between human-associated fecal sources (Fig. 1-8), and between non-human fecal sources (Fig. 1-9). The results of TRFLP fingerprinting whole bacterial communities in sources, reference material and spiked reference material are presented as plots resulting from PCA analysis which is a multivariate statistical approach commonly used to compare microbial community composition from one sample to another. By TRFLP-PCA, sewage, septic solids, and human sources appeared dissimilar to one another (Fig 1-10). Human fecal material appeared more similar to raccoon, cat, gull, sewage, and dog than to septic solids. Addition of sewage to reference waters at all concentrations could be detected by TRFLP as seen in the PCA plot by the grouping of spiked waters close to sewage while the source water (1 sample, Rattlesnake Canyon) was separate (Fig. 1-11). Addition of septic solids to reference waters (whether ocean, Arroyo Burro Lagoon, Veronica Springs water) did not alter the reference water microbial community fingerprint except at the highest concentration (5%) at which Veronica Springs was spiked (Fig. 1-12). Signals of sewage and septic solids added to soil and sediments were lost in these matrices, i.e. the fingerprints of spiked soils and sediments grouped in PCA space with background matrices (Fig. 1-11 and 1-12). Spiking Veronica Springs water with dog and cat feces caused this water to resemble the fecal sources; however, dog and cat fecal sources were not discernible from one another (Fig. 1-12). Ocean water samples were mostly distinct (Fig. 1-11 and 1-12). The addition of human feces to ocean water could be discerned (Fig. 1-11). Neither septic solids, cat nor dog source materials could be discerned from background ocean water (Fig. 1-12). Water sampled from just downstream of the Westside Drain (Bohnett Park) did not appear similar to any fecal sources including sewage (Fig. 1-11). Water from Arroyo Burro Creek and Arroyo Burro Lagoon all appeared similar to one another (Fig. 1-12).

1.4 Conclusions

Based on the results of this initial portion of the overall project, it appeared that the HGM method is useful for discovering the presence of sewage in environmental waters in Santa Barbara. However, assaying for only presence / absence provides limited power for further relating contamination to potential sources. Thus, transition to a qPCR method for the remainder of this project is advisable. This type of method became available at the end of the Phase I research, and thus was adopted at that time (see Chapter 2 and later chapters). The inability of the HGM approach for distinguishing septic solids is likely related to the source of waste. As discussed in the next chapter, a conclusion regarding the septic solids used in this first Phase was that it was unlikely to have represented septage. The solids that are pumped from septic tanks are accumulated over a several year period, are highly anaerobic, and are compartmentalized physically such that distinct microbial communities could be selected. On the other hand, the liquid effluent from septic tanks is what enters the leach field and thus better represents the type of sample that could contaminate ground and surface waters. Thus, early in Phase II, as described in Chapter 2, septage was directly sampled from a septic tank associated with a public restroom and that sample was used for further comparison to environmental waters.

The most promising evidence that TRFLP would be useful for this ongoing research was its ability to discern sewage-contaminated environmental waters from uncontaminated waters with low overall sewage contamination. The lowest targeted concentration of sewage spiked in to Rattlesnake Canyon water was 0.01%. Based on the FIB content of the sewage (Table 1-5), this lowest spike resulted in an *Enterococcus* concentration of 169 MPN/l00 mL and an *E. coli* concentration of 1274 MPN/100 mL. The *Enterococcus* concentration is within the range of the single-sample limit for beach ocean water quality in California (104 MPN/100 mL), as prescribed by AB411 (http://www.dhs.ca.gov/PS/DDWEM/beaches/pdfs/AppendixA.pdf). Whether or not TRFLP could discern the presence of sewage in spiked creek water at lower concentrations was not tested here, but this result is still promising.

TRFLP also reasonably reflected the presence of dog and cat fecal spikes into VSW (Fig. 1-12). However, dog and cat fecal material appeared very similar to one

another in this PCA plot, which could reflect either a high degree of similarity between these taxa, or a high degree of variability in the fecal microflora between host animals of the same taxa. When individual animal fecal communities were compared within the dog and cat host groups, indeed the two taxa appeared to overlap significantly (not shown). However, explaining variations between individuals in their fecal microbial communities was beyond the scope of this research. Still, the implication is that sewage contamination can be resolved against background microbial communities to a level that is relevant to water quality, and that the relative influence of other sources can be distinguished on the basis of TRFLP data subjected to multivariate statistical analysis.

Where TRFLP failed to reveal the presence of spiked contamination was in soils and sediments. It is difficult to say exactly why the soils and sediments retained the signatures of their original microbial communities even after being spiked to fairly high levels with fecal materials. One explanation is that spiked communities were lost by sorption to soil or sediment organomineral complexes. Alternatively, predation or lysis removed a substantial portion of the community that was added. Whatever the exact cause, this issue in the research was not further addressed yet would be interesting for future investigation.

In Conclusion, the overall use of DNA-based methods in Phase I was promising enough for future microbiological water quality investigations such that Phase II included their use. Thus, at the time that this work was originally reported, it was decided to proceed with Phase II activities by applying DNA-based methods to other key locations in the Mission Creek watershed that are listed in the scope of work, including the Mission and Laguna lagoons. Also, it was decided to carefully plan and possibly re-scope the originally planned Arroyo Burro Lagoon studies in Phase II, particularly in light of any new results generated from repeated studies using actual septage. Additional ideas for future activities that did not become priorities in the Phase II research included spiking Bohnett Park creek water with sewage from El Estero and from a nearby, relevant, manhole in the area then determining the ability to discern sewage in this semi-urban water; spiking and studying recovery of sewage from Bohnett Park sediments, and intensively studying the area downstream of the Westside Drain, as discussed in the Phase II scope of work, through one or two 24-hour periods to capture potential

relationships of water contamination to temporal patterns in wastewater generation in the area.

1.5 References

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TABLE 1-1: Liquid RECIPES and SAMPLES for Mission Creek

Description	Sample ID	Sewage mL[1]	Human Stock mL[2]	Racoon Stock mL[3]	Gull Stock mL[4]	RCW mL[5]	Ocean Water mL[6]	IDEXX dilution
Human + RC water	RCW-H	0	2	0	0	1998	0	1:1000
Human + Ocean water	MCO-H	0	2	0	0	0	1998	1:1000
Sewage + RC water (5%)	RCW-S5	100	0	0	0	1900	0	1:10,000
Sewage + RC water (1%)	RCW-S1	20	0	0	0	1980	0	1:1000
Sewage + RC water (0.1%)	RCW-S01	2.0	0	0	0	1998	0	1:100
Sewage + RC water (0.01%)	RCW-S001	0.20	0	0	0	1999.8	0	1:10
High Sewage+ Raccoon + Gull	RCWSGR-H	10	0	0.50	0.50	1989	0	1:1000
Low Sewage+ Raccoon + Gull	RCWSGR-L	4	0	0.80	0.80	1994.4	0	1:1000
Sewage + Gull	RCWSG	10	0	0	1	1989	0	1:1000
Sewage + Raccoon	RCWSR	10	0	1	0	1989	0	1:1000
Bohnett Park Water	BPW	0	0	0	0	2000	0	none
Gull Stock	gs							1:10,000
Raccoon Stock	rs							1:10,000
Human Stock	hs							1:10,000
Sewage	sewage							1:100,000
Ocean Water	MCO							none
RC Water	RCW							none
Sewage stock,[7] 1%	ss							1:1000

[1]Sewage: undiluted sewage from influence of El Estero WWTP, well mixed.
[2]Human stock: 2 g composited human feces into 100 mL container plus 99 mL RC water (assuming human feces are 50% water); feces were from Cottage Hospital with roughly equivalent portions from 3 individuals, well composited.
[3]Raccoon stock: 2 g composited feces from multiple (>3) animal hosts (CARE wildlife rescue agency) added into 100 mL container plus add 99 mL RC water.
[4]Gull stock: 2 g composited feces from multiple (>3) animal hosts (baited at East Beach) added into 100 mL container plus add 99 mL RC water.
[5]RCW: Rattlesnake Canyon creek water.
[6]Ocean: ankle deep water from surf zone at East Beach.
[7]Sewage stock (1%) consisting of 10 mL sewage from El Estero WWTP.
plus 990 mL RC creek water.

TABLE 1-2: Solid RECIPES and SAMPLES for Mission Creek[1]

Description	Sample ID	Sewage Stock mL	Soil g[2]	Sediment g[3]	Racoon Stock mL	Gull Stock mL	Creek Water mL	IDEXX Dilution
Sewage + Soil	RCsoil-S	1	2	0	0	0	98	1:1000
Sewage + Sediments	RCsed-S	1	0	2	0	0	98	1:1000
High Sewage + Animals + Sediments	RCSGR-H	0.5	0	2	0.25	0.25	98	1:1000
Low Sewage + Animals + Sediments	RCSGR-L	0.2	0	2	0.40	0.40	98	1:1000
Sewage + Gull + Sediments	RCsedSG	0.5	0	2	0	0.5	98	1:1000
Sewage + Raccoon + Sediments	RCsedSR	0.5	0	2	0.5	0	98	1:1000
Gull fecal material	gull							
Raccoon fecal material	raccoon							
Human fecal material	human							
Bohnett Park soil[4]	BPsoil							1:1000
Bohnett Park sediment[5]	BPsed							1:1000
Rattlesnake Canyon soil	RCsoil							1:1000
Rattlesnake Canyon sediment	RCsed							1:1000

[1]Labels as in Table 1 unless otherwise noted.
[2]Soil from Rattlesnake Canyon (RC) near creek bank.
[3] Sediments from RC creek bottom.
[4]Soil from Bohnett Park near creek crossing.
[5]Sediments from Bohnett Park near creek crossing.

TABLE 1-3: Liquid RECIPES and SAMPLES for Arroyo Burro[1]

Description	Sample ID	SS[2] diluted mL	Dog Stock mL	Cat Stock mL	ABL[3] mL	VSW[4] mL	OW[5] mL	IDEXX dilution
SS, diluted	septic solids	1000						1:100,000
OW	ABO						1000	none
OW + SS (1%)	ABO-S	10	0	0	0	0	990	1:1000
OW + SS + cat + dog, high	ABOSCD-H	5	0.25	0.25	0		994.5	1:1000
OW + SS + cat + dog, low	ABOSCD-L	2.0	0.4	0.4	0		997.2	1:1000
OW + SS + cat	ABOSC	5	0	0.5	0		994.5	1:1000
OW + SS + dog	ABOSD	5	0.5	0	0		994.5	1:1000
ABL	ABL	0	0	0	1000	0	0	1:10,000
ABL + SS	ABL-S	10	0	0	990	0	0	1:10,000
ABL + SS, particles	ABL-Sp **DELETED**							
ABL+ SS + cat+ dog, high	ABLSCD-H	5	0.25	0.25	994.5		0	1:10,000
ABL + SS + cat+ dog, low	ABLSCD-L	2.0	0.4	0.4	997.2		0	1:10,000
ABL + SS + cat	ABLSC	5	0	0.5	994.5		0	1:10,000
ABL + SS + dog	ABLSD	5	0.5	0	994.5		0	1:10,000
AB Creek water[6]	ABCW							1:10
VSW	VSW					1000		none
VSW + SS, 5%	VSW-S5	50				950		1:10,000
VSW+ SS, 1%	VSW-S1	10				990		1:1000
VSW + SS, 0.1%	VSW-S01	1				999		1:100
VSW + SS, 0.01%	VSW-S001	0.1				999.9		1:10
VSW + SS + cat + dog, high	VSWSCD-H	5	0.25	0.25	0	994.5	0	1:10,000
VSW + SS + cat + dog, low	VSWSCD-L	2.0	0.4	0.4	0	997.2	0	1:10,000
VSW + SS + cat	VSWSC	5	0	0.5	0	994.5	0	1:10,000
VSW +SS + dog	VSWSD	5	0.5	0	0	994.5	0	1:10,000
Cat stock	cat							1:100,000
Dog stock	dog							1:100,000
SS stock (1%)[7]	seps							1:1000

[1]Labels as in Tables 1-1 and 1-2, unless otherwise noted.
[2]Septic solids from MarBorg Quarantina facility pumping truck tank, diluted in sterile water to match sewage (approximately 1000 mg/L) concentration as per Methods.
[3]ABL: Arroyo Burro Lagoon water
[4]VSW: Veronica Springs water
[5]OW: Ocean water from Hendry's Beach near the outlet of ABL
[6]Arroyo Burro Creek water at Cliff Drive
[7]SS: septic solids stock solution consisting of a 1% mixture in VSW

TABLE 1-4: Solid RECIPES and SAMPLES for Arroyo Burro[1]

Description	Sample ID	SS Stock mL	Soil g	Sediment g	Dog Stock mL	Cat Stock mL	Creek Water mL	IDEXX Dilution
cat fecal material	Cat	2 g material + 99 mL sterile Nanopure water						1:100,000
dog fecal material	Dog	2 g material + 99 mL sterile Nanopure water						1:100,000
Arroyo Burro creek sediment	ABCsed	2 g material + 99 mL sterile Nanopure water						1:10
Veronica Springs soil	VSWsoil	2 g material + 99 mL sterile Nanopure water						1:10
Veronica Springs soil + SS	VSWsoil-S	1	2		0	0	98	1:100
Veronica Springs sediment	VSWsed	2 g material + 99 mL sterile Nanopure water						1:100
Veronica Springs sediment + SS	VSWsed-S	1		2			98	1:100
Arroyo Burro Lagoon sediment	ABLsed	2 g material + 99 mL sterile Nanopure water						1:1000
Arroyo Burro Lagoon sediment + SS	ABLsed-S	1		2			98	1:1000

[1]Labels as in Tables 1-1 through 1-3, unless otherwise noted.

TABLE 1-5: Fecal Indicator Bacterial Concentrations for Mission Creek Phase I Samples, Spiked Samples and Fecal Sources. IDs are as per Tables 1-1 and 1-2.

Sample ID	Total Coliform		*E. coli*		Enterococcus	
	Dilution 1:x	MPN/100ml	Dilution 1:x	MPN/100ml	Dilution 1:x	MPN/100ml
sewage	100000	92080000	100000	12740000	100000	1690000
BPW	NONE	>2419.6	NONE	39.9	NONE	>2419.6
MCO	NONE	613.1	NONE	161.6	NONE	63.3
MCO-H	1000	1986300	1000	1732900	1000	27200
RCW	NONE	43.5	NONE	<1	NONE	<1
RCW-H	1000	1986300	1000	2419600	1000	9700
RCW-S5	10000	909000	10000	318000	10000	20000
RCW-S1	1000	198900	1000	53700	1000	5200
RCW-S01	100	24890	100	7030	100	1090
RCW-S001	10	2755	10	884	10	132
RCWSGR-H	1000	648800	1000	387300	1000	9500
RCWSGR-L	1000	547500	1000	517200	1000	6200
RCWSG	1000	146700	1000	41400	1000	1000
RCWSR	1000	275500	1000	248100	1000	32300
ss	1000	325500	1000	61600	1000	5200
hs	10000	>24196000	10000	>24196000	10000	>24196000
gs	10000	17329000	10000	11199000	10000	1046000
rs	10000	>24196000	10000	>24196000	10000	>24196000
BPsed	1000	<1000	1000	<1000	1000	<1000
BPsoil	1000	3100	1000	<1000	1000	1000
RCsed	1000	<1000	1000	<1000	1000	<1000
RCsed-S	1000	3000	1000	<1000	1000	<1000
RCSGR-H	1000	1986300	1000	1986300	1000	105400
RCSGR-L	1000	>2419600	1000	>2419600	1000	122300
RCsedSG	1000	87800	1000	46200	1000	1000
RCsedSR	1000	>2419600	1000	>2419600	1000	101900
RCsoil	1000	1000	1000	<1000	1000	<1000
RCsoil-S	1000	12100	1000	1000	1000	<1000

TABLE 1-6: Fecal Indicator Bacterial Concentrations for Arroyo Burro Creek Phase I Samples, Spiked Samples and Fecal Sources. IDs are as per Tables 1-3 and 1-4.

Sample ID	Total Coliform		*E. coli*		Enterococcus	
	Dilution 1:x	MPN/100ml after dilution	Dilution 1:x	MPN/100ml after dilution	Dilution 1:x	MPN/100ml after dilution
Septic solids	100000	10000000000.0	100000.0	100000	100000	<100000
ABO	NONE	NONE	NONE	84.2	NONE	68.7
ABO-S	1000	1000000.0	1000.0	1000	1000	1000
ABOSCD-H	1000	1000000.0	1000	16100	1000	<1000
ABOSCD-L	1000	1000000.0	1000.0	27900	1000	<1000
ABOSC	1000	1000000.0	1000.0	<1000	1000	<1000
ABOSD	1000	1000000.0	1000	27500	1000	<1000
ABL	NONE	NONE	NONE	272.3	NONE	172.2
ABL-S	10000	100000000.0	10000.0	<10000	10000	<10000
ABLSCD-H	10000	100000000.0	10000.0	<10000	10000	<10000
ABLSCD-L	10000	100000000.0	10000.0	10000	10000	<10000
ABLSC	10000	100000000.0	10000.0	10000	10000	<10000
ABLSD	10000	<10000	10000.0	<10000	10000	<10000
ABCW	NONE	NONE	NONE	50.4	NONE	19.9
VSW	NONE	NONE	NONE	10.9	NONE	21.3
VSW-S5	10000	<10000	10000	<10000	10000	<10000
VSW-S1	1000	<1000	1000	<1000	1000	<1000
VSW-S01	100	10000.0	100	<100	100	<100
VSW-S001	10	100.0	10	10	10	20
VSWSCD-H	10000	100000000.0	10000	31000	10000	<10000
VSWSCD-L	10000	100000000.0	10000	63000	10000	<10000
VSWSC	10000	<10000	10000	<10000	10000	<10000
VSWSD	10000	100000000.0	10000	20000	10000	<10000
cat stock	100000	10000000000.0	100000	2130000	100000	<100000
dog stock	100000	10000000000.0	100000	141360000	100000	6700000
SS stock	1000	1000000.0	1000	1000	1000	1000
ABCsed	10	100.0	10	259	10	108
VSWsoil	10	>24196	10	52	10	275
VSWsoil-S	100	10000.0	100	100	100	100
VSWsed	100	10000.0	100	<100	100	100
VSWsed-S	100	10000.0	100	1080	100	100
ABLsed	1000	<1000	1000	<1000	1000	<1000
ABLsed-S	1000	1000000.0	1000.0	1000	1000	<1000

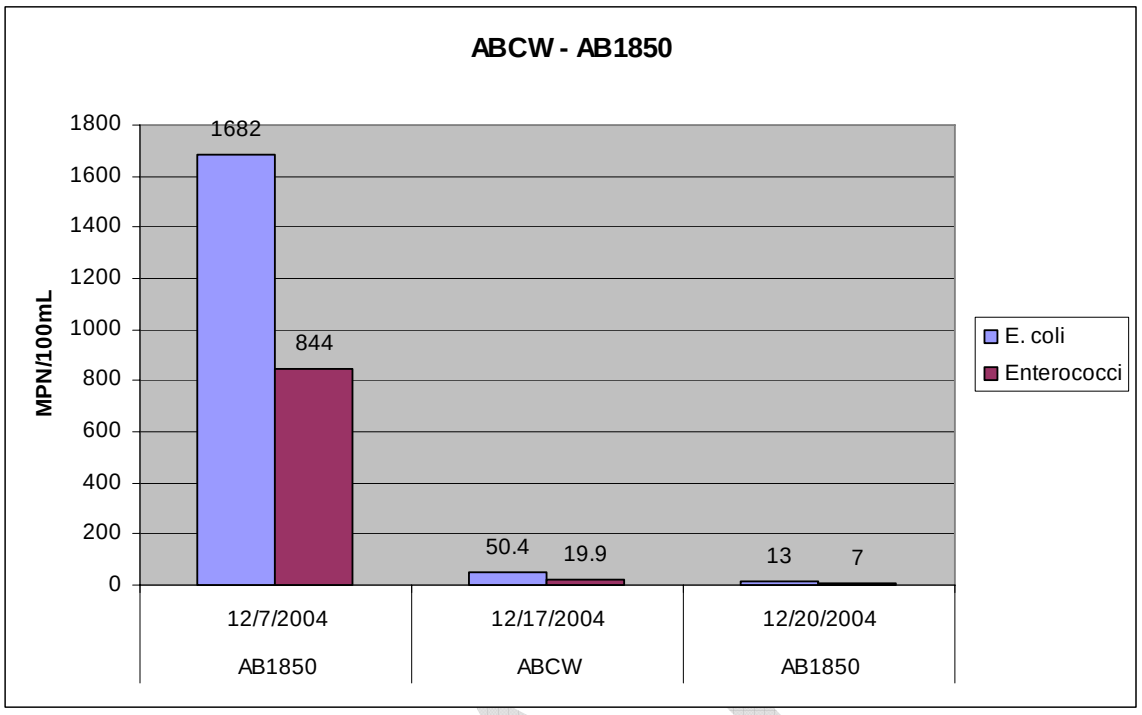

FIGURE 1-1: Comparison of Phase I FIB for Arroyo Burro Creek Water (ABCW) to City Analyses for the Same Site Before and After Phase I Sampling.

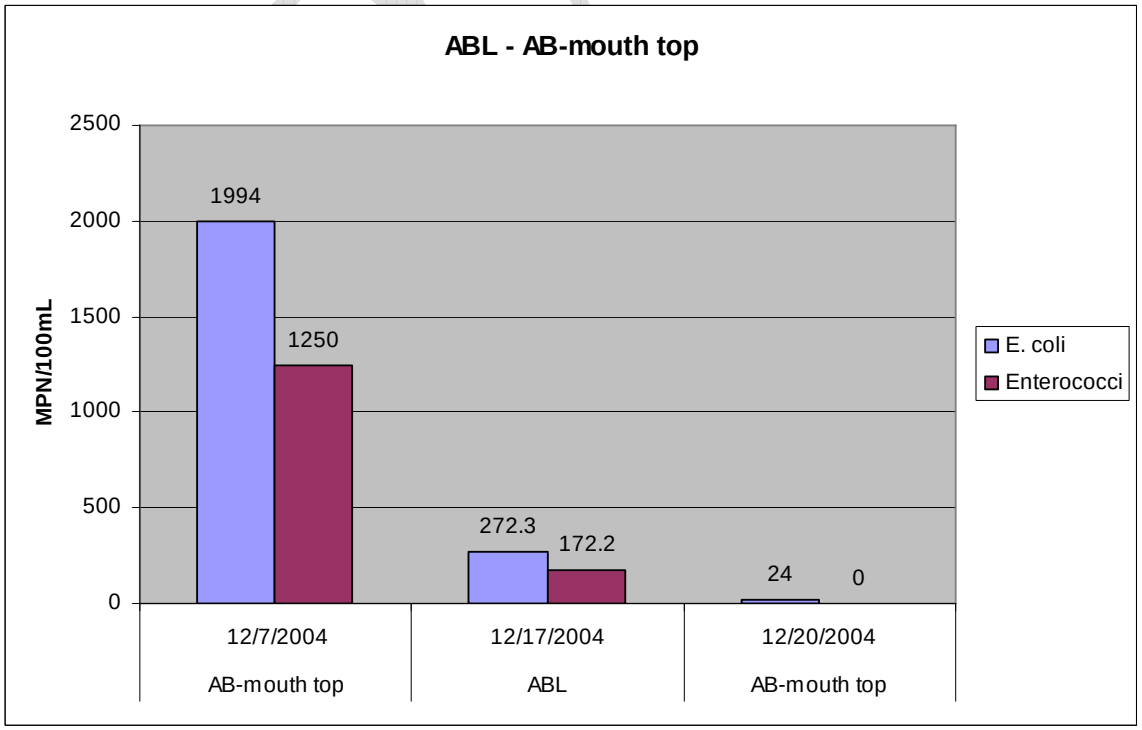

FIGURE 1-2: Comparison of Phase I FIB for Arroyo Burro Lagoon (ABL) Water to City Analyses for the Same Site Before and After Phase I Sampling.

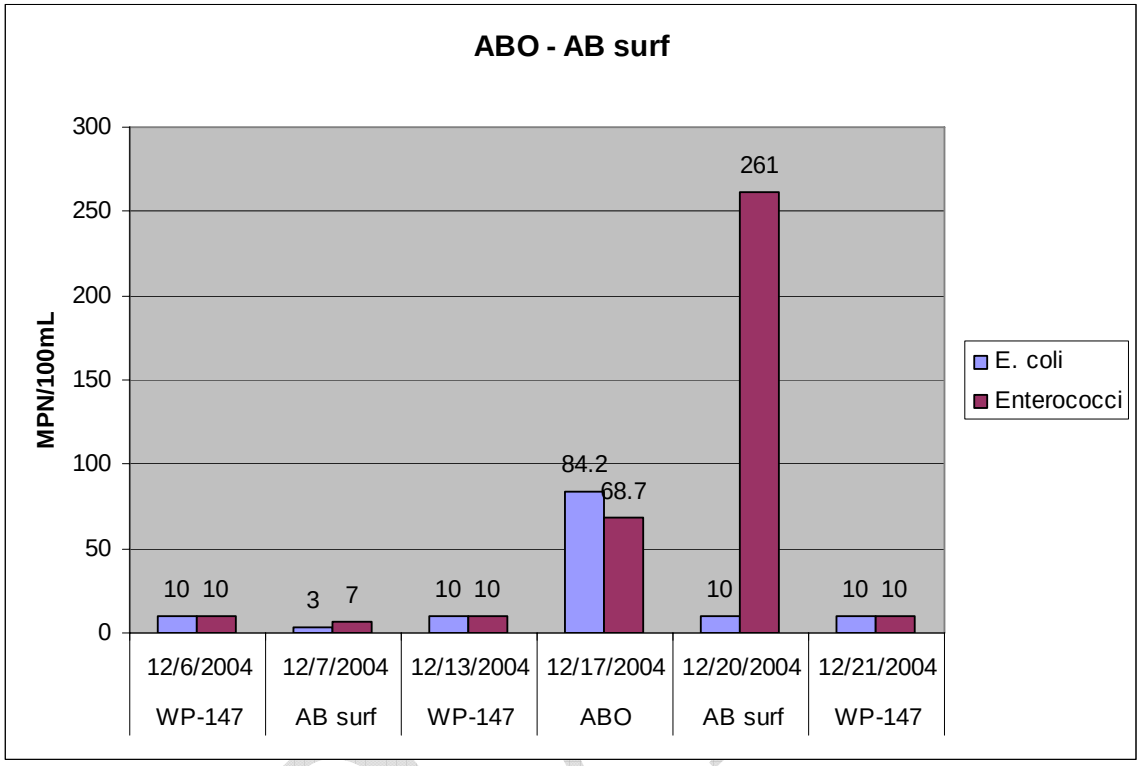

FIGURE 1-3: Comparison of Phase I FIB for Arroyo Burro Ocean (ABO) Water to City and County Analyses for Hendry's Beach Surf Zone Waters.

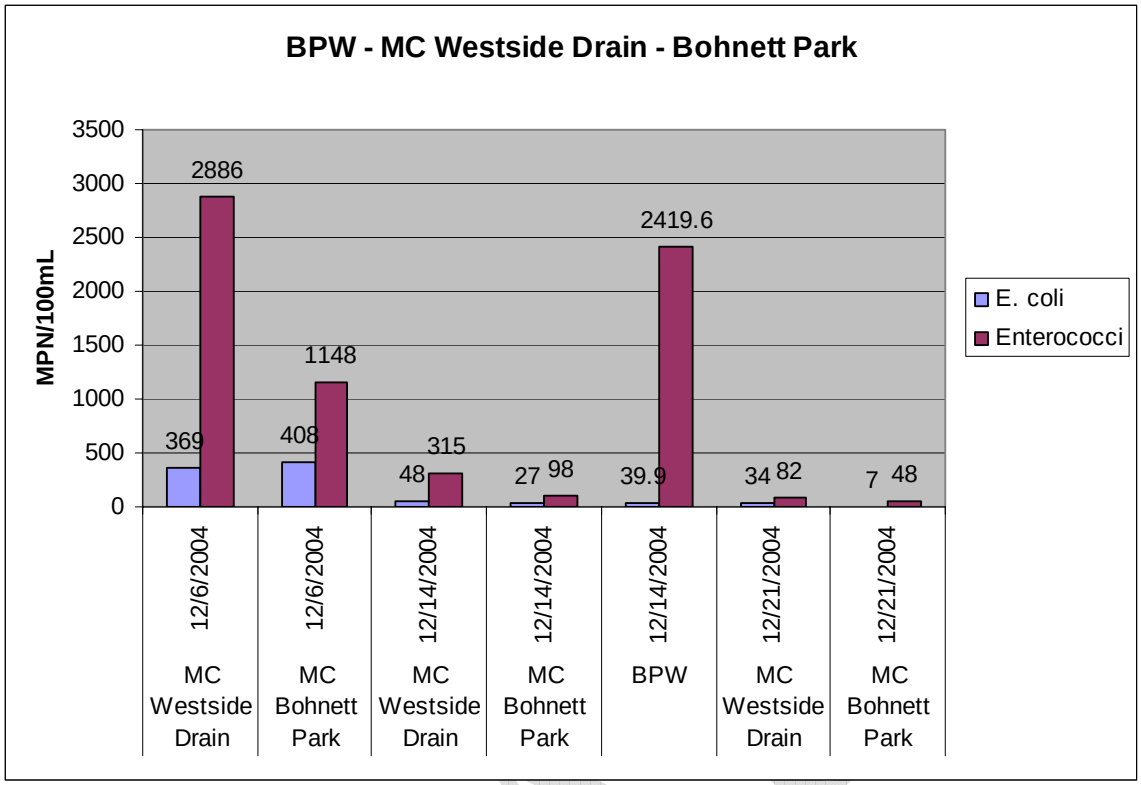

FIGURE 1-4: Comparison of Phase I FIB for Mission Creek (MC) Samples on 12/14/2004 to City Analyses for the Same Sites on Dates Preceding and Following Phase I Sampling.

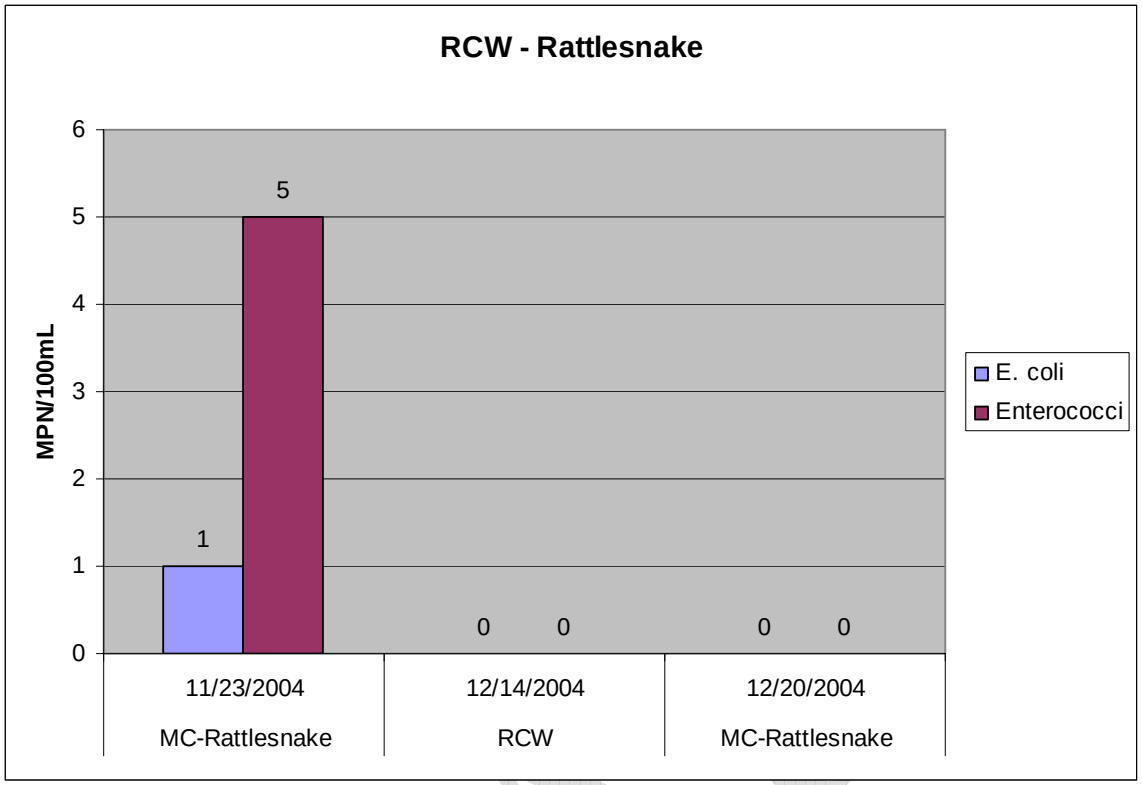

FIGURE 1-5: Comparison of Phase I FIB for Rattlesnake Creek Water (RCW) to City Analyses Before and After Phase I Sampling.

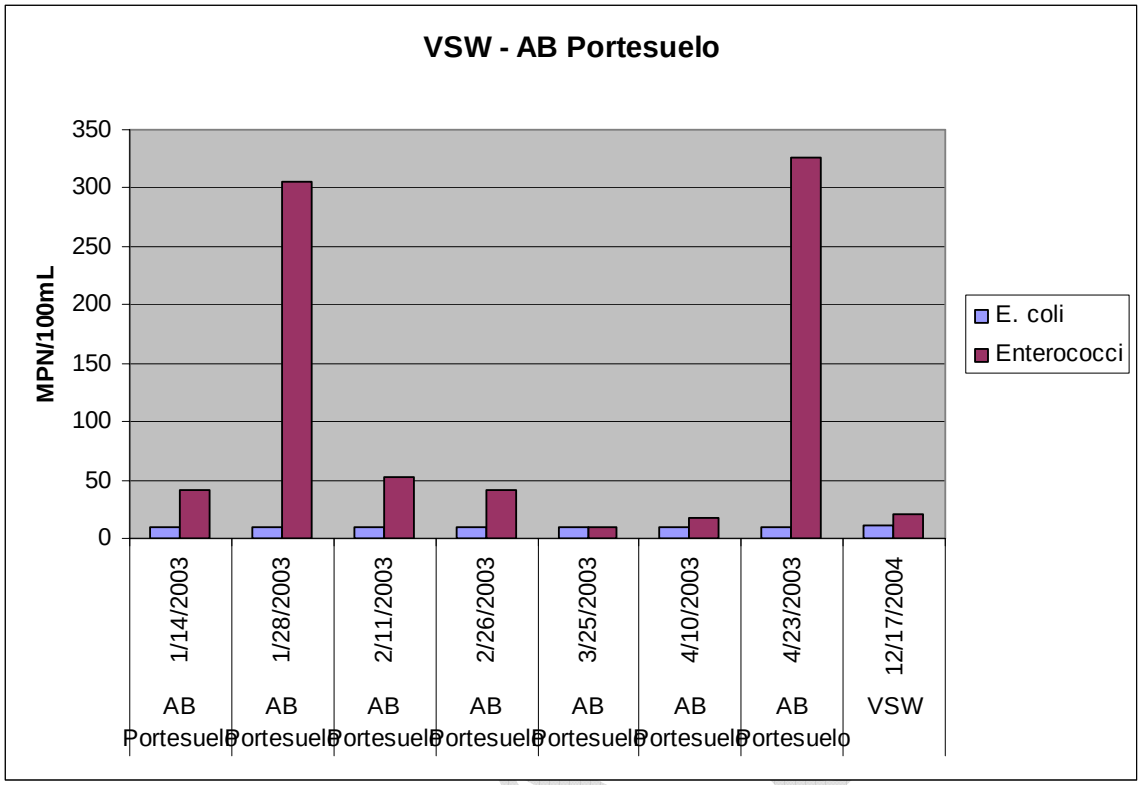

FIGURE 1-6: Comparison of Phase I FIB for Veronica Springs Water (VSW) to City Samples Analyzed Before Phase I at Arroyo Burro (AB) Portesuelo

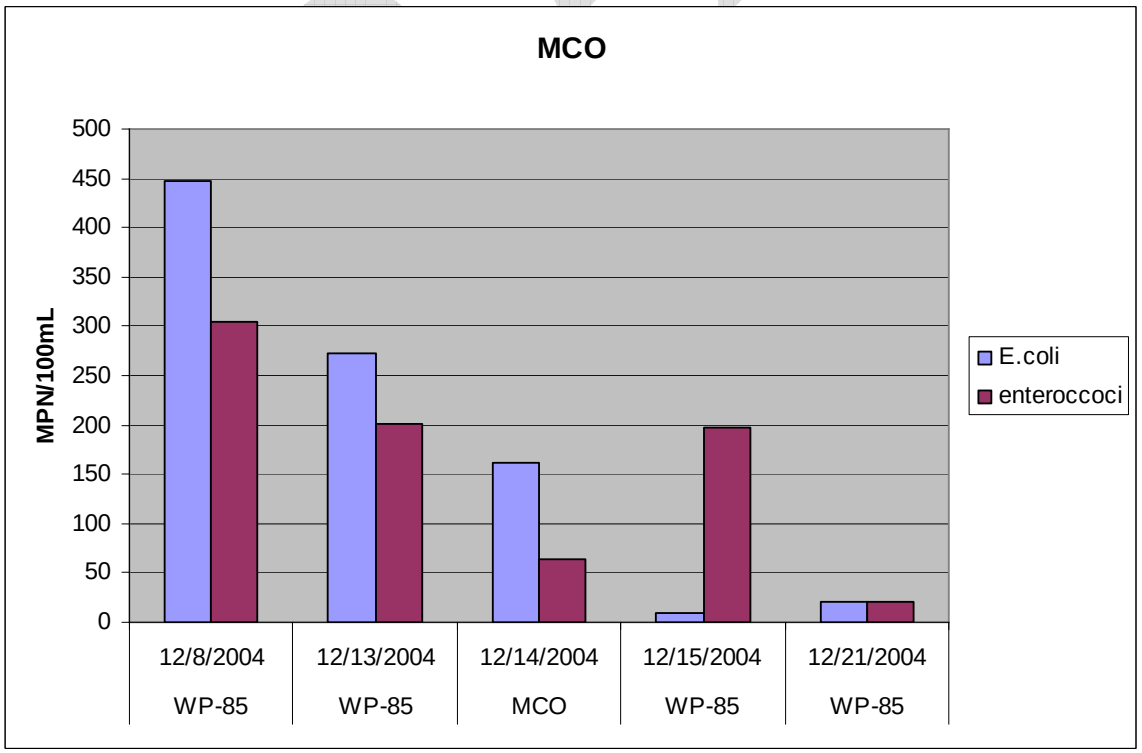

FIGURE 1-7: Comparison of Phase I FIB for Mission Creek Ocean (MCO) at East Beach to County Samples Analyzed Before and After Phase I at the Proximate County Station (WP-85)

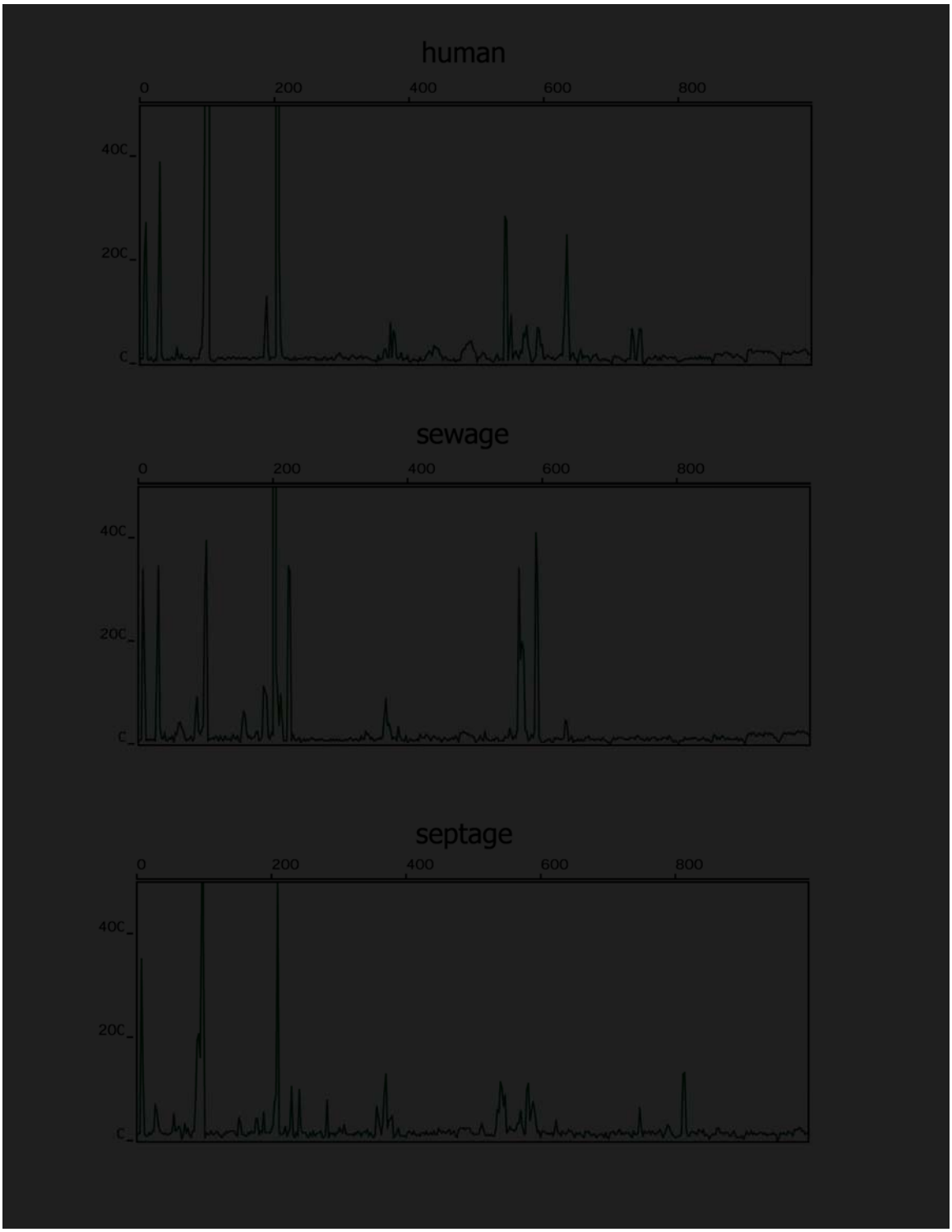

FIGURE 1-8: Electropherograms from TRFLP Analyses of Human-Associated Fecal Sources in Phase I. Note that "septage" was actually septic tank solids in Phase I.

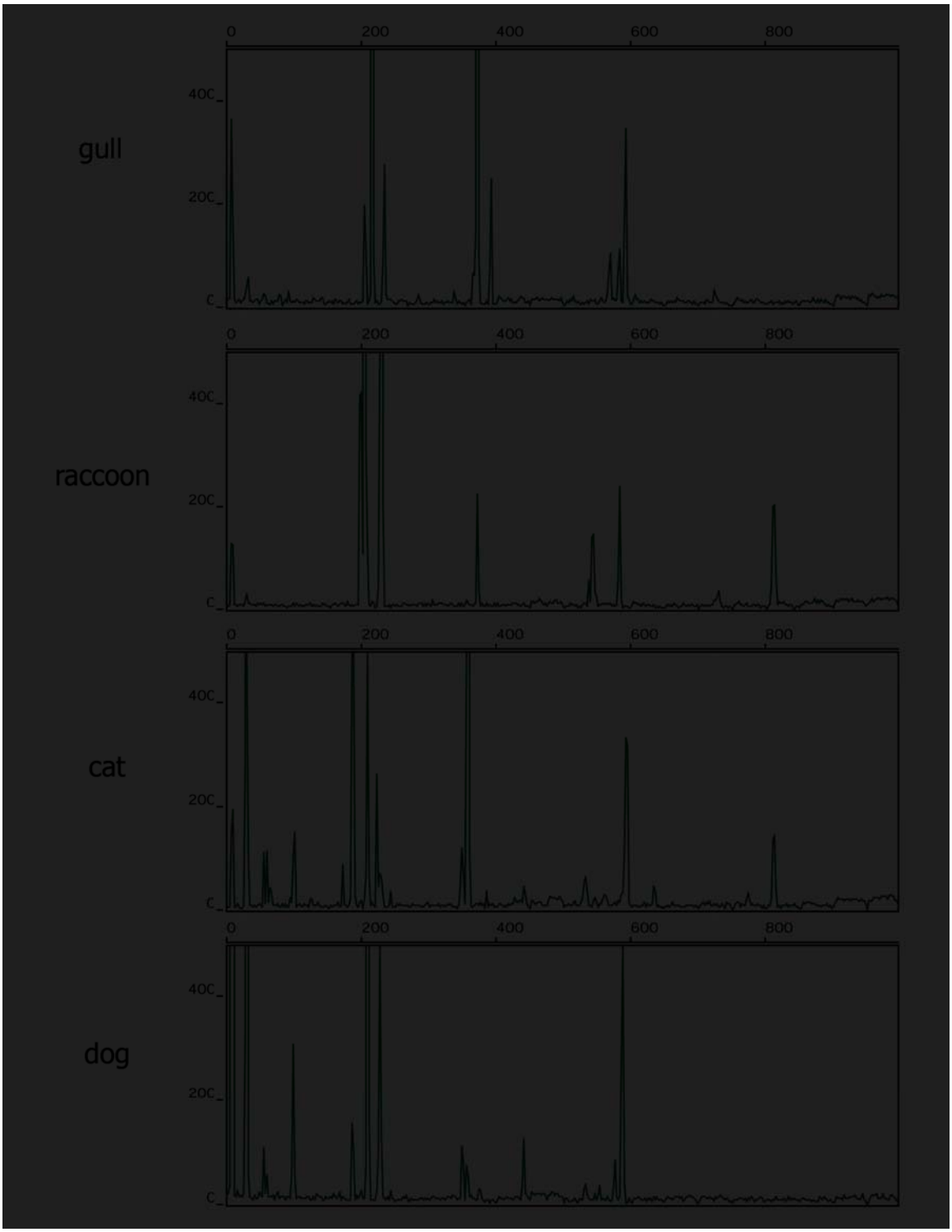

FIGURE 1-9: Electropherograms from TRFLP Analyses of Non-Human Fecal Sources in Phase I. Sources are Described in the Methods.

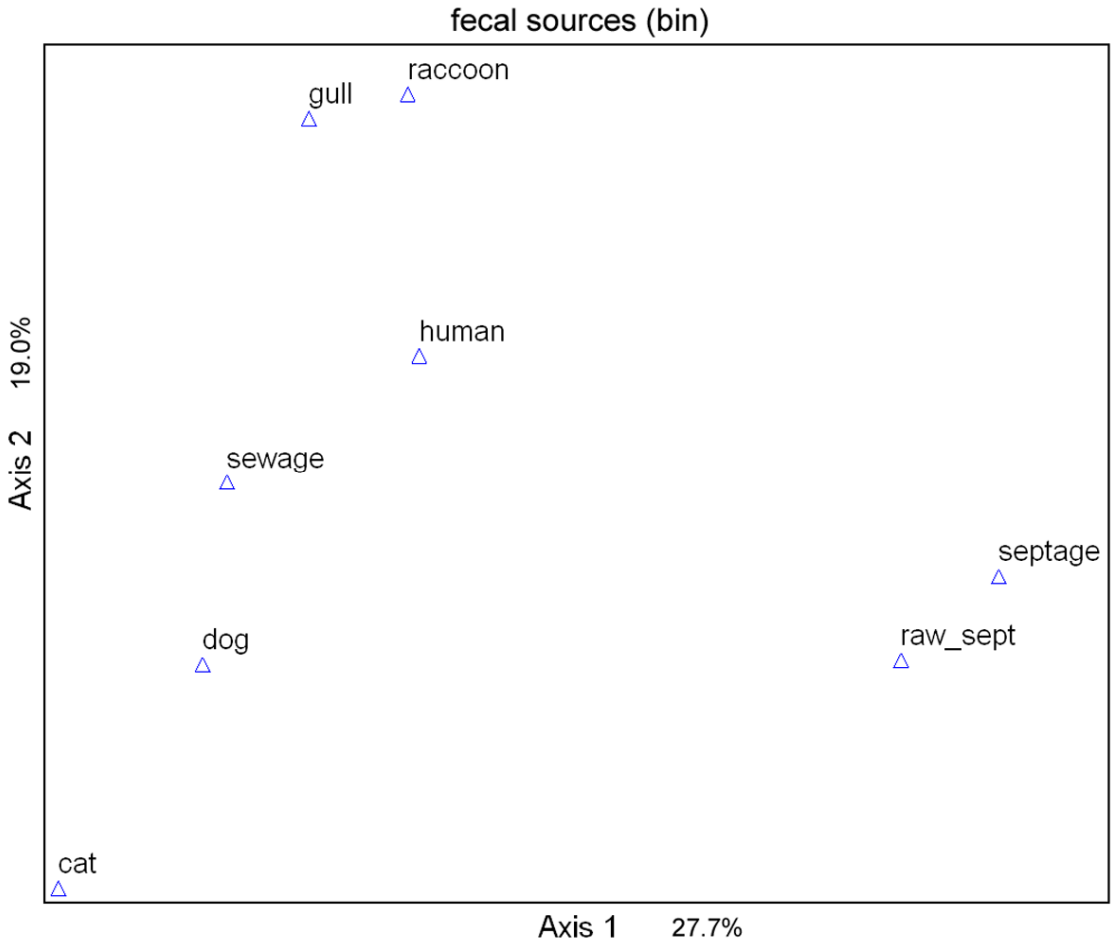

FIGURE 1-10: PCA Plot from TRFLP Analysis of Fecal Source Samples in Phase I.

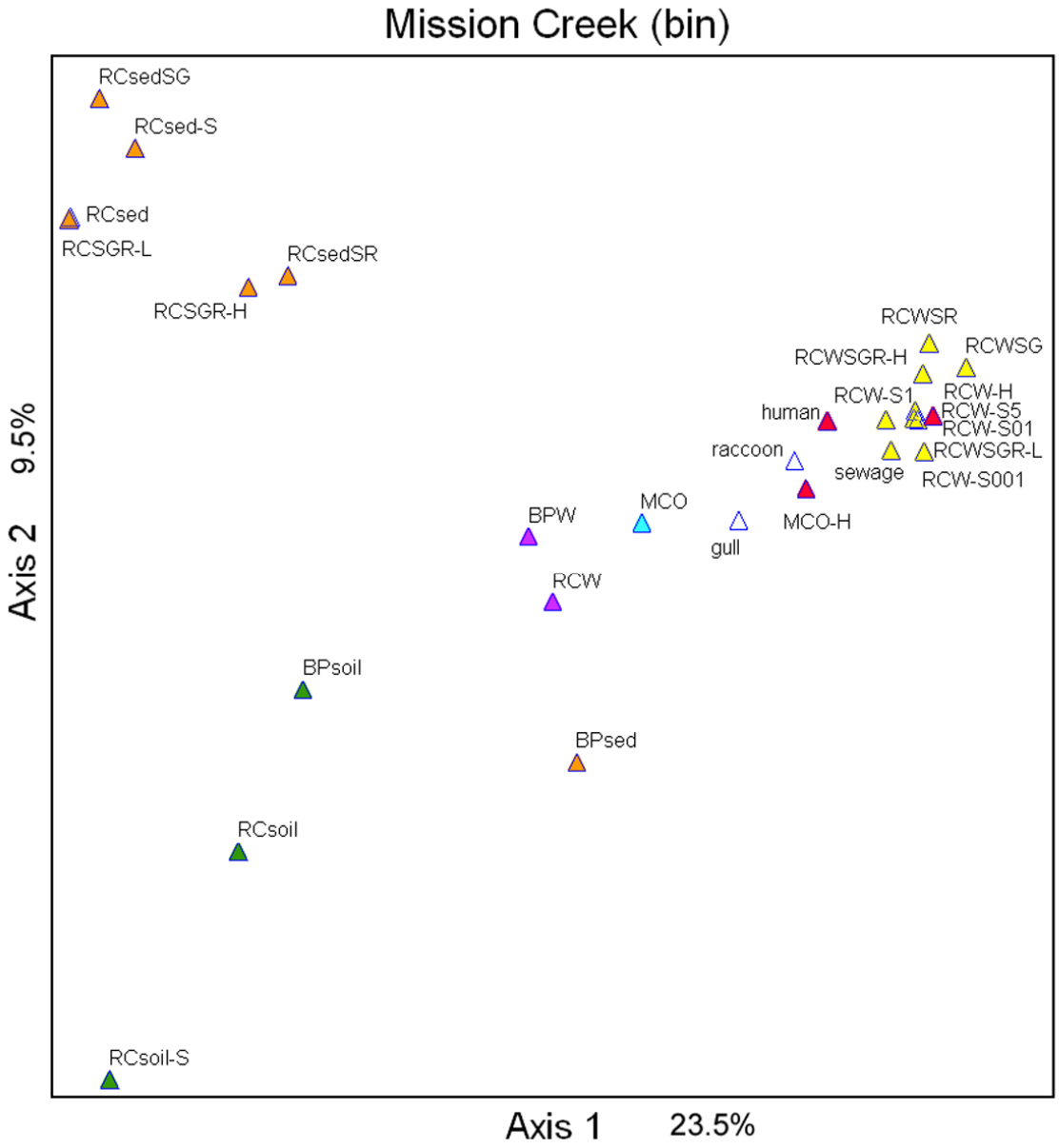

FIGURE 1-11: PCA Plot from TRFLP Analysis of Mission Creek Samples in Phase I. Sample IDs are provided in Tables 1-1 and 1-2.

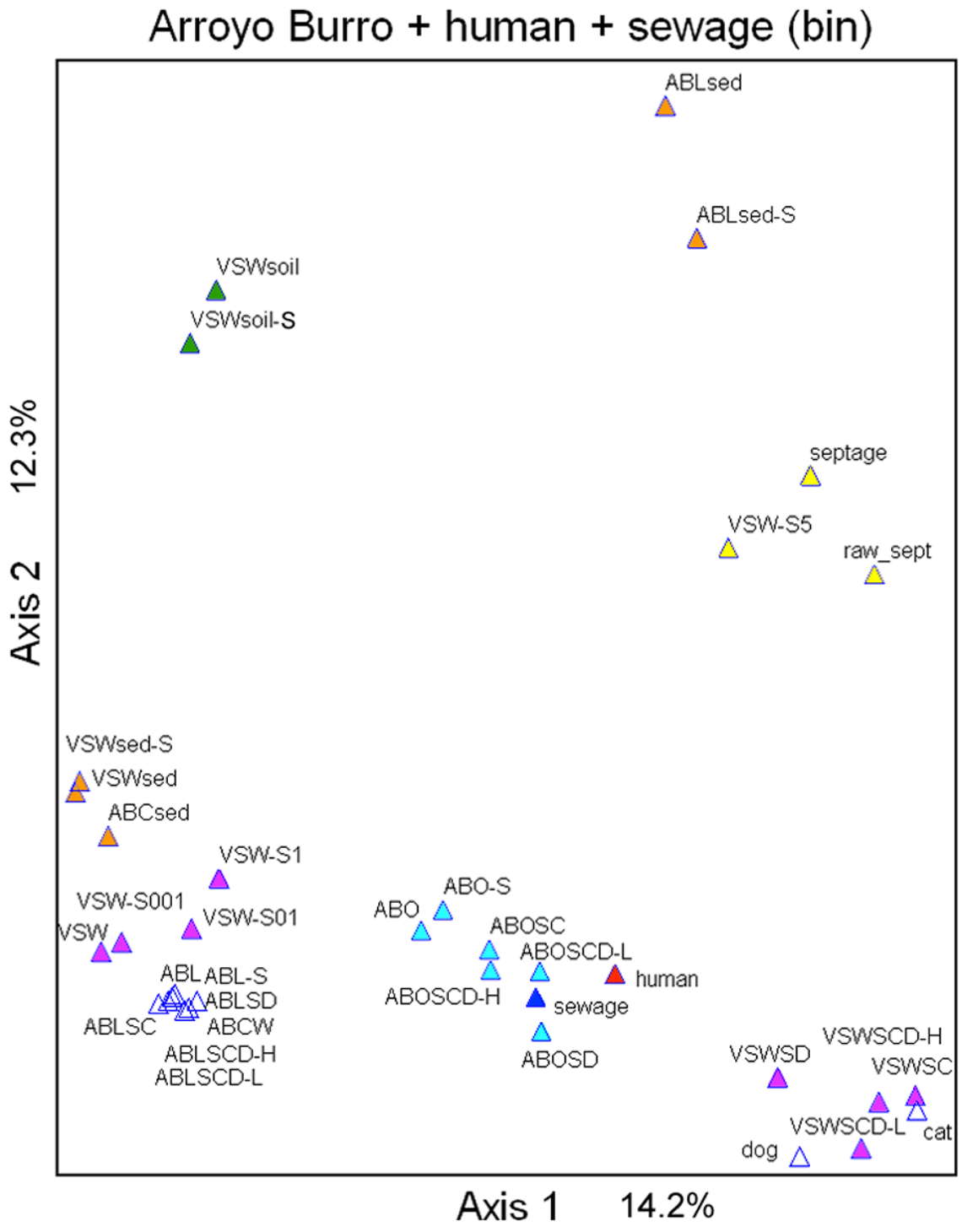

FIGURE 1-12: PCA Plot from TRFLP Analysis of Arroyo Burro Creek Samples in Phase I. Sample IDs are provided in Tables 1-3 and 1-4. Note that septage = septic solids, as described in the Methods.

Chapter 2: Microbiological Water Quality and Fecal Source Tracking in Two California Coastal Creeks (Phase IIA, Phase IIC, and the Phase III Haley Drain Tracking and Hope Drain Tracking Studies)

2.1 Introduction

Coastal water quality is typically assessed by quantifying indicators of fecal pollution: total coliform, either fecal coliform or *Escherichia coli*, and enterococci (17, 47). These indicators are not pathogens and are not specific to humans, but epidemiology studies link them to swimmer illnesses (5, 12, 19, 20, 22, 40, 46, 48), particularly in areas where there are known "point" sources of pollution (4, 6, 22, 48). Unfortunately, most pollution sources leading to beach closures are "unknown" (14). Further, finding the human-associated sources by quantifying indicator bacteria alone is difficult because indicator bacteria are not specific to humans, survive in the natural environment (1, 13, 23, 24, 31, 37) to different degrees compared to pathogens (52), and can become non-culturable (30, 44). The first two issues lead to costly false positive-based beach closures, and the third issue can lead to dirty waters being posted as clean, thus not protecting public health.

In urban areas, sources of fecal indicators and pathogens may be near-shore including leaking sewer lines (3, 33), natural features such as decaying wrack (49), algae (51), and coastal marshes with enterococci-generating waterfowl (21). Septic systems in unsewered areas may also be important. (15, 38, 50). Away from shore, urban development overall contributes to relatively high fecal indicator bacterial concentrations(10), and urban infrastructure may be implicated including storm drains in the Houston area (34) and in urban southern California (16, 26, 36, 45) that discharge high loads of fecal indicator bacteria and pathogens even in dry-weather. However, high concentrations of fecal indicator bacteria arise from eroded sediments in channels and storm drains (16, 36) and possibly soil from banks and beyond (41, 42). Thus, the simple

association of fecal indicator bacteria with urban infrastructure like storm drains reveals little about either the presence or source of specific wastes.

To determine if human waste is present in fecal indicator-contaminated waters including dry weather drainage, DNA can be extracted and amplified for a gene marker that is specific to human fecal-associated *Bacteroides* (2). This method offers considerable power in discerning the presence of human waste because it is highly specific and culture-independent. Further the human gene marker (HGM) can be quantified in a qPCR assay, and thus target concentrations compared to potential sources of human waste such as sewage (39) which allows estimating the relative quantity of human-associated contamination. However, multiple animal hosts can simultaneously affect water quality and thus either other DNA-based waste-specific markers or other DNA-based approaches, such as microbial community profiling (7, 11, 27), are required to assign a relative importance of humans versus other potential host animals as fecal contamination contributors.

In this study, waters from urban reaches in two Santa Barbara, CA creeks were surveyed for evidence of human-associated waste and bacterial communities were analyzed for their relatedness to various waste sources. The longitudinal transects spanned fresh to lagoon to coastal ocean waters with sites that were historically high in dry-weather fecal indicator bacterial concentrations. The questions motivating this study included: is human waste present?; what are the origins?; and where does it arise in the system? Importantly, human waste was evidenced throughout both transects and concentrations were highest in each transect at a storm drain discharging flow during dry weather. While the exact points of entry into the storm drain systems were not definitively determined, this study clearly demonstrates that discharges from urban storm drain infrastructure contribute to human-waste contamination of surface waters and that, in this setting, their influence was stronger than other fecal sources.

2.2 Materials and Methods

2.2.1 Study sites and sampling

Study sites were in lower Mission Creek and Arroyo Burro watersheds, in the City of Santa Barbara, CA. Due to pathogen pollution, each watershed contains a creek that is listed on the Clean Water Act Section 303-(d) List of Water Quality Limited Segments (Mission Creek and Arroyo Burro Creek), and both terminate at beaches frequently posted with warnings against recreational use based on fecal indicator bacteria (FIB) levels that Santa Barbara County measures weekly. Using this historical ocean FIB data, along with creek FIB measurements taken routinely by the City of Santa Barbara, nine to ten study sites were selected along the lower, semi-urbanized portions of each watershed. This included urban storm drains that flow into the creek year round, and creek discharge into lagoons and the ocean. Working from downstream to upstream, each site was sampled once a day for three consecutive days at approximately the same time and similar tidal states in the summer of 2005. All sampling was carried out during the dry weather period, with no rainfall for at least forty-eight days prior.

The Mission Creek watershed covers about 7,203 acres. The dominant land uses are rural residential and high-density residential, while recreational, institutional and agriculture are relatively scarce (8). Focusing primarily on lower Mission Creek, nine sites were sampled from June 28 - 30, 2005 (Fig. 2-1): ocean discharge (M1), Laguna lagoon (M2), Laguna channel (M3), Mission lagoon (M4), Mission Creek at Montecito St. (M5), Haley drain discharge (M6), Mission Creek at Haley St. (M7), Old Mission Creek confluence into Mission Creek (M8), and Westside drain (M9). Flow measurements were obtained for the three day study from the USGS for Mission Creek at Rocky Nook Park (http://nwis.waterdata.usgs.gov/nwis/measurements/?site_no=11119745&amp ; average = 0.015 cubic meters per second (cms)), which is upstream from downtown Santa

Barbara and the sampling sites, and from the UCSB LTER project (http://www.lternet.edu/sites/sbc/) for site M5 (average = 0.016 cms).

The Arroyo Burro watershed encompasses approximately 6,311 acres. The dominant land uses are open space and medium-density residential uses, while commercial/industrial, office complex and agriculture are scarce (8). Focusing mainly on lower Arroyo Burro Creek, ten sites were sampled from August 23 – 25, 2005 (Fig. 2-1): ocean discharge (A1), Arroyo Burro lagoon mouth (A2), above Arroyo Burro lagoon (A3), Mesa Creek/drain (A4), Arroyo Burro Creek (ABC) at Cliff Dr. (A5), ABC below Las Positas Creek (A6), ABC at Hidden Valley Park (A7), ABC downstream of Hope drain (A8), Hope drain discharge (A9), and Las Positas Creek at Modoc Rd. (A10). Flow measurements were obtained from the UCSB LTER project for site A5 (average = 0.49 cfs).

Water samples (approximately 2 L) were grabbed using a sterile beaker, passed through Miracloth (Calbiochem/EMD Biosciences, San Diego, CA) to remove large debris, and stored on ice until transport back to the lab where they were processed within 6 hours. Dissolved oxygen, temperature, and salinity were measured in the field with the YSI Model 85 handheld meter (YSI Inc., Yellow Springs, OH), and pH was measured in the lab with a Corning pH meter 430 (Corning, NY).

DNA from samples collected in this study were compared to DNA collected from putative fecal sources collected during Phase I, with the exception that septage was newly collected. Also, additional samples of raw sewage were collected during the course of this study from the El Estero WWTP, partly for the purposes of evaluating the temporal variations in microbiological water quality of WWTP influent. As described in Chapter 1, fecal sources were chosen in consultation with staff from the City of Santa Barbara Creeks Division based on sources deemed to be most relevant for each watershed. For Mission Creek, relevant sources were sewage, human, gull, and raccoon; for Arroyo Burro, sources were sewage, septage, dog, and cat. Raw sewage samples were collected from the influent at the El Estero Wastewater Treatment Plant (Santa Barbara, CA) on four separate dates (10/12/04, 12/14/04, 6/30/05, and 10/24/05). A septage sample was obtained from MarBorg Industries (Santa Barbara, CA) during a septic tank pumping event at the Santa Barbara Botanic Garden (9/8/2005). As described in Chapter 1, human

feces were from 3 individuals at a local hospital lab (12/14/2004). Gull feces were collected on two separate occasions (12/14/2004 and 3/28/2006) by baiting onto clean, plastic tarps. Feces from a minimum of 3 individual gulls were scraped with Samplit™ Sterile Scoop & Container System disposable sampling scoops (Sterileware®, Bel-Art Products, Pequannock, NJ) and composited into the attached vessel. Raccoon feces from 3 healthy individuals from the Santa Barbara Wildlife Care Network were similarly scooped from individual cages (12/14/2004) and composited. Dog feces were acquired (12/17/2004) and composited as above from 3 healthy individuals (male Shepard mix, female Retriever, female miniature Dachshund), each from a separate household. Cat feces were similarly obtained (12/17/2004) from 3 healthy individuals (all male, mixed breeds) of two separate households.

2.2.2 Fecal indicator bacteria

Based on historical FIB data, sterile Nanopure water was added to dilute a portion of each sample (100 mL of total volume). Using commercial reagents and methods (IDEXX Laboratories, Westbrook, MA), total coliform, *E. coli* and enterococci were quantified by adding the appropriate reagent packet (Colilert or Enterolert) to the diluted sample, mixing, sealing in a Quanti-Tray®/2000, and incubating for 24 hours (at 35ºC and 41ºC respectively). Samples were then analyzed for color change and/or fluorescence and quantified via the most probable number (MPN) table provided by the manufacturer.

2.2.3 DNA extraction

The UltraClean™ Water DNA Kit (MO BIO Laboratories, Carlsbad, CA) was used to collect bacteria from the water and extract the DNA. Water samples, sewage and septage samples were vacuum filtered through 0.22 μm filters until the collected volume was filtered or the filters reached the point of refusal. The filters were then stored at -20ºC until extraction. DNA was extracted according to the kit's protocol, and followed by ethanol precipitation to further concentrate the DNA. Fecal sample DNA was extracted using approximately 0.25 g wet weight feces in the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) following the kit's protocol. Total DNA was quantified

using the Quant-iT™ PicoGreen® dsDNA kit (Molecular Probes/Invitrogen) using the supplied lambda DNA as the standard. Sample DNA was stored at -20ºC until analysis.

2.2.4 16S PCR and TRFLP

Genes encoding 16S rRNA were PCR amplified from purified DNA samples using universal primers 8F hex (fluorescently labeled) and 1389R as described before (11). PCR products were purified with the QIAquick PCR purification kit (QIAGEN, Valencia, CA), and ca. 300 ng of purified DNA was digested with *Hha*-I (New England BioLabs, Ipswich, MA). After inactivation of the restriction enzyme by heating (65°C, 20 min), the lengths of fluorescently labeled fragments were determined with an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the Genomics Technology Support Facility (Michigan State University).

The individual peak heights of the terminal restriction fragments (TRFs) were normalized to the percentage of total height for that sample, and peaks with a relative height of less than 1% were discarded. The TRFs were aligned using the crosstab macro written by Dr. C. Walsh (http://www.wsc.monash.edu.au/~cwalsh/treeflap.xls). The aligned data was imported into the software Primer (version 6, Primer-E Ltd, U.K.), and a Bray-Curtis similarity matrix was calculated both before and after transforming the peaks to presence/absence. Non-metric Multi-Dimensional Scaling (MDS), with 100 random restarts, was used to ordinate the similarity data (35). The normalized peak data was also used to calculate species richness, species evenness, and the Shannon diversity index with the use of PC-ORD (version 4, MJM Software, Gleneden Beach, OR).

2.2.5 Human-specific Bacteroides qPCR

A standard was first created by amplifying DNA extracted from sewage influent samples via a conventional PCR method with human-specific HF 183 *Bacteroides* primers (2). The PCR products were pooled, then purified and quantified in the same manner as the 16S PCR products previously mentioned. This standard was then serially diluted and run on every qPCR plate.

Sample DNA was quantitatively-PCR (qPCR) amplified using a published method for the human-specific HF 183 *Bacteroides* 16S rRNA genetic marker with SYBR® Green I detection (39). Primer design and reaction conditions were identical to the published method except for the instrument used (Bio-Rad iCycler iQ®, Hercules, CA) and the addition of fluorescein (Eurogentec, Belgium) which was necessary to enable dynamic well factor collection and data optimization on the iQ system. Each plate contained, in triplicate, a tenfold dilution of the sewage *Bacteroides* standard, ranging from 3.8×10^7 to 3.8×10^1 human-specific *Bacteroides* markers per microliter of DNA extract, a no-template control, and the samples to be analyzed. To allow comparisons, each plate was standardized by adjusting the baseline threshold position until the Ct values for the standard dilutions were less than 3% from run to run. The resulting sample Ct values were then used to calculate the number of human-specific *Bacteroides* markers per liter of sample filtered, and the triplicate values for each sample were averaged. Any replicates that did not amplify, or amplified after the lowest sewage standard, were treated as a zero value in the calculations. To ensure correct target amplification, a melt curve was run and verified for each sample.

2.2.6 Statistical analysis

For each watershed, IDEXX results (*E. coli* and enterococci), TRFLP results (species richness, evenness, and diversity), and the human-specific *Bacteroides* qPCR results were each further analyzed separately via One-Way ANOVA. For the IDEXX results, only measurements within the proper dilution range (no ">" or "<" values) were utilized in statistical analysis. Due to the unequal variances of the means across the sites, the Dunnett's T3 pairwise comparison test was used in lieu of other analysis of variance post hoc tests which assume equal variances (i.e. Tukey's HSD). All statistics were carried out in SPSS version 12 (SPSS Inc., Chicago, IL). Select TRFLP-MDS plots were analyzed via the ANOSIM (analysis of similarity) and SIMPER (similarity percentage analysis) options within the Primer software.

2.2.7 Storm drain tracking

Based on the results from the initial three-day studies in each watershed, two urban storm drains (Haley drain in Mission Creek, and Hope drain in Arroyo Burro) had human-specific *Bacteroides* markers detected and their quantities were generally highest along the sampling transects. To try and pinpoint where the human waste is entering each system, several drain tracking sampling events were undertaken. On August 2, 2005, sites M4, M5, M6 (Haley drain) were re-sampled, followed by sampling upstream in the storm drain system itself (the Continuous Deflective Separation Unit (CDS) just upstream in the pipe from where the drain discharges into the creek, and two manholes).

From August 15 – 17, 2006, the Haley drain system was examined again, this time each site was sampled once a day for three consecutive days, and more sites upstream in the drain system were selected:

- Haley drain diversion (M6)
- CDS unit
- manhole @ Haley & Chapala
- manhole @ Chapala & Cota
- manhole @ Chapala & Ortega
- drop inlet @ Chapala & Ortega
- sump 1 @ Paseo Nuevo in parking garage
- sump 2 @ Paseo Nuevo in parking garage
- sump 3 @ Paseo Nuevo in parking garage
- Nordstrom's basement sump

The Hope drain system in the Arroyo Burro watershed was also sampled once a day for three consecutive days from September 5 – 7, 2006:

- Hope drain diversion (A9)
- drop inlet near Starbucks (La Cumbre Plaza)
- side drain near Red Robin (La Cumbre Plaza)
- manhole @ Ritz Camera & Vons (La Cumbre Plaza)
- manhole near Cingular (La Cumbre Plaza)
- drop inlet near La Salsa (Five Points Shopping Center)

- bus stop near Spectrum Gym (La Cumbre Rd. & State St.)

Sampling, extraction and analyses were similar to before, except an ISCO 6712 sampler (Teledyne Isco, Inc., Lincoln, NE) was used in the Hope drain system, and only IDEXX and human-specific *Bacteroides* qPCR analyses were performed.

2.3 Results

2.3.1 Physical and microbiological site characteristics

For both transects, DO was relatively highest in two lagoons (M4 and A2) and relatively low at only a few locations (M3, M5, M6, A10); water temperature was relatively constant except for an apparent low in the ocean at M1 and an apparent high in the drain at M6, pH varied little, and salinity varied expectedly along the ocean to freshwater gradient (Tables 2-1 and 2-2).

In the Mission Creek study area, the average concentrations of *E. coli* and enterococci increased through the urban portion of the creek, reaching the highest level at site M6 (Haley Drain that flows year-round)(Table 2-3). However, due to the high daily variation in sample values, post-hoc testing (Dunnett's T3) revealed no significant difference between the sites for either FIB test. Similar results were found for *E. coli* measured in the Arroyo Burro study area, with the highest average values at sites A9 (another urban storm drain with year-round flow) and A10 (Table 2-4), again with no significant differences detected. This was not the case for enterococci measured at Arroyo Burro. The same two sites had the highest average values, but site A8 was significantly different from A2, A5 and A7, and site A1 was significantly different from A3 and A6 (Table 2-4). Relationships between DO and fecal indicator bacteria were suggested in that two of the Mission Creek sites that had the lowest DO concentrations (M5 and M6; Table 2-1) showed relatively higher concentrations of *E. coli* (Table 2-3). Similarly, the Arroyo Burro site with the lowest DO concentration (A10; Table 2-2), also had a relatively higher *E. coli* concentration (Table 2-4).

2.3.2 Bacterial community richness and evenness

There appears to be an urban influence on species richness, evenness, and diversity in the Mission Creek study area. These three indices were highest at the most upstream location site M9; decreased through site M7; spiked at the urban storm drain site M6; then decreased downstream to the ocean (Table 2-3). For species richness, site M9 was significantly different from every site except M8; and site M6 from site M1 and M7. Evenness was significant only for site M9 from M2 and M3. Species diversity had similar results to richness, with site M9 again significantly different from every site except M8; and site M6 from M1, M5, and M7 (Table 2-3).

There was a similar influence in the Arroyo Burro study area. The three indices started high at sites A9 (urban storm drain) and A10; decreased through site A5; spiked at site A4 which drains the western portion of the Mesa; then decreased downstream to the ocean (Table 2-4). For species richness, site A9 was significantly different from A5, A6 and A7; site A4 from A1, A5, A6 and A7; and site A2 from A5, A6 and A7. There was no significant difference between the sites for evenness. For species diversity, site A9 was significantly different from A5 and A6; site A4 from A5 and A6; and site A2 from A5 and A6 (Table 2-4).

2.3.2 TRFLP-MDS

TRFLP-MDS using one enzyme was able to sufficiently separate the bacterial communities in the different fecal sources (Fig. 2-2). A second enzyme (*Msp*-I) was also tested in combination but did not make an appreciable increase in separation, so only *Hha*-I was used in analysis (data not shown). The Mission Creek lagoon and creek samples, when plotted with the fecal sources of interest, grouped closer to sewage than to either gull or raccoon, while the ocean samples appeared distinct (Fig. 2-3). ANOSIM analysis revealed that each group (sewage/creeks/lagoons/urban drains, ocean, gull, and raccoon) were significantly different from each other ($P < 0.001$). Human was not utilized in the MDS plots with samples because our fecal source (sick individuals from a hospital environment) was most likely not representative of the human sources we might see in the Mission Creek watershed. Plotting all Mission Creek samples with sewage

resulted in separate clusters of sewage; creeks and Laguna lagoon; urban drains; Mission lagoon; and ocean samples, arranged in the direction of flow (Fig. 2-4).

A similar separation of sewage, urban drains, creeks and lagoon, and ocean occurred when plotting all Arroyo Burro samples with sewage, with the additional separation of the Mesa Creek samples from the other creeks (Fig. 2-5). All Arroyo Burro watershed samples grouped closer to each other and sewage than the other fecal sources of interest (septage, dog, cat), and again the ocean samples appeared distinct (Fig. 2-6). Similar to the Mission Creek results, these Arroyo Burro samples also had significant separation between the groups (sewage/creeks/lagoon/urban drains, ocean, dog, cat, and septage) ($P < 0.001$).

SIMPER analysis examines the role of individual species/peaks in contributing to the closeness of samples within a group, and the separation between two groups. For the Mission Creek samples, the clustering groups similar to Figure 2-3 were used: creeks, lagoons, and urban drains were considered one group, while sewage, ocean, raccoon and gull were each separate groups. Sewage was pulled out as a separate group from the creek/drain/lagoon group to evaluate its similarity to the other groups. Table 2-5 presents the "characteristic" peaks of each group, and the percent similarity between the groups. The creek/drain/lagoon group was less similar (< 4% and < 6%, respectively) to gull or raccoon fecal sources, and more similar to sewage (> 22%, the highest similarity reported in this analysis).

An additional SIMPER analysis was run using the above groups but also separating Haley drain from the creeks/lagoons/urban drains group to evaluate its similarity to the remaining groups (Table 2-6). The highest similarity percentage was between Haley drain and the creek/drain/lagoon group (> 29 %), followed by Haley drain and sewage (> 26%).

Upon closer examination of the characteristic peaks reported in Table 2-6, bubble plots, based on Figure 2-4, were created for each of the six key sewage peaks. Of particular interest was peak #202, which was also a key peak for Haley drain (Figure 2-7). Although, due to rounding differences during the TRFLP alignment process, peaks 205 and 565 in sewage could be the same as peaks 206 and 564 in Haley drain. Peak #202 was found in the Haley drain (M6) and Westside drain (M9) samples on all three

days; at Mission lagoon (M4) and Montecito (M5) on two of the days; and at Haley creek (M7) and OMC into MC (M8) on one of the sampling days.

SIMPER analysis was also run for the Arroyo Burro samples, based on the clustering of groups in Figure 6. Similar to Mission Creek, SIMPER was run with sewage as a separate group from the creek/drain/lagoon group (Table 2-7) and also with Hope drain as a separate group (Table 2-8).

Bubble plots based on Figure 2-5 were created for each of the six key sewage peaks. Similar to the Mission Creek samples, peak #202 was again a key peak present in both sewage and Hope drain, and peaks #205 and 565 were present as well. Peak #202 was found in the Mesa Creek/drain (A4) and Hope drain (A9) samples on all three days, and at downstream of Hope drain (A8) and the lagoon mouth (A2) on two of the sampling days (Fig. 2-8). In comparison, peak #205 was found in the majority of sites (Fig. 2-9). Peak #565 was more selective and similar to the results for peak #202. Peak #565 was found in Site A8 and A9 samples on all three days; at site A4 on two days; and at Las Positas Creek @ Modoc (A10) on one of the sampling days (Fig. 2-10).

2.3.3 Human-specific Bacteroides qPCR

All fecal sources were analyzed via human-specific *Bacteroides* qPCR. As expected, gull, raccoon, cat, and dog fecal samples did not have any human-specific *Bacteroides* markers amplify. Human fecal (average = 2.9E+07 markers/g wet, SE = 1.1E+06), septage (average = 3.9E+09 markers/L, SE = 1.2E+08) and all sewage samples (3 different dates, average = 7.8E+09, SE = 2.4E+08) did amplify, and were within published ranges (39). Our limit of quantification (LOQ), based on our averaged sewage results, was approximately 0.0001% sewage (= in the order of 10E+3 to 10E+04 markers/L).

In Mission Creek, Haley drain (M6) had the highest number of markers detected on all 3 days (Fig. 2-11), and was significantly different from the other sites (One-Way ANOVA with Dunnett's T3, $\alpha = 0.05$). The values for Site M6 from the 3 days varied from 1.5E+05 to 1.7E+07, indicating strong temporal variation in the human-specific *Bacteroides* signal at this drain.

In Arroyo Burro, Hope Drain (A9) and the point just downstream (A8) consistently had the highest numbers of markers detected (Fig. 2-12), but there was no statistically significant differences between the sites. Sites A1-A3 and A10 had detectable markers on the first day only, also indicating temporal variation in this system.

Plotting the human-specific *Bacteroides* results along with the *E. coli* and enterococci results from IDEXX, suggested a log-log relationship between them (Figs. 2-13 & 2-14).

For each watershed, simple and logarithmic regressions were run to determine if there was a correlation between the number of human-specific *Bacteroides* markers and *E. coli* or enterococci. Though displaying similar trends, for Mission Creek, neither log *E. coli* vs. log *Bacteroides* (P = 0.156, R = 0.552), log enterococci vs. log *Bacteroides* (P = 0.056, R = 0.695), nor log *E. coli* vs. log enterococci (P = 0.074, R = 0.661) had significant correlations. The Arroyo Burro samples also had the same visual trends, but for these samples log *E. coli* vs. log *Bacteroides* (P = 0.005, R = 0.724) and log *E. coli* vs. log enterococci (P = 0.0003, R = 0.840) were significantly correlated, while log enterococci vs. log *Bacteroides* (P = 0.058, R = 0.538) was not.

2.3.4 Storm drain tracking

In the Mission Creek watershed, only site M6 (Haley drain) had detectable levels of human-specific *Bacteroides* markers on every sampling occasion (n = 10), resulting in an average of 2.9E+07 markers/L and standard error of 6.7E+06 (= 0.38% average and 0.09 standard error when expressed as a percentage of our measured and averaged sewage samples). The actual values from sampling period to sampling period varied highly: from 1.7E+05 to 2.0E+08 markers/L (= 0.002 to 2.6% of sewage) overall, and from 2.8E+05 to 2.0E+08 markers/L (= 0.004 to 2.6% of sewage) in a single day (Fig. 2-15).

For the 2006 Haley drain tracking sampling events, fecal pollution as measured by human-specific *Bacteroides* qPCR was concentrated in the three most downstream locations; while targets were also detected at one upstream location during the 2005 sampling event (Fig. 2-16). No targets were detected further upstream in the shopping

center complex, nor was the exact origin of the human fecal signal determined. However, the concentrations of signal in these three locations suggest the importance of emphasizing these sites in future studies.

In the Arroyo Burro watershed, Hope drain (A9) also displayed high temporal variation, with the average number of markers ranging from below the limit of quantification to 1.9E+08 markers/L (= < 0.0001 to 2.5% of sewage) in less than 2 hours in a single day, when it was sampled again due to a significant flow increase (Fig. 2-17). The Hope drain tracking sampling events did not reveal the location of where human fecal material is entering the storm drain system (Fig. 2-18). However, upstream sources are implied when even only one of three qPCR assays show positive results.

The FIB results for both systems once again varied highly from day to day. For the Haley drain system, sump #2 at Paseo Nuevo consistently had the lowest levels for all three FIB indices; and the highest levels of *E. coli* and enterococci were concentrated in the three most downstream sites, with the exception of the Nordstrom's basement sump on August 17, 2006 (Table 2-9). In general, the Hope drain system had higher FIB results than the Haley system, although no consistent pattern emerged for any of the FIB indices. On September 6, 2006, when the Hope drain diversion was sampled twice in the same morning, the FIB levels dramatically increased from around 10 or less to more than 19863 MPN/L (Table 2-10).

2.4 Conclusions

This study sought to determine if human waste was an important source of dry weather contamination in two urban and suburban Santa Barbara, California creeks where FIB were historically high, and to determine where human waste was originating in the creek environments. Through a combination of approaches, i.e. specific DNA-based assessment of human waste and bacterial community profiling for inferring the importance of other contamination sources, it was clear that human waste was present throughout each system and that other waste sources that were examined were less influential on microbiological water quality. Further, human waste was entering these coastal creeks from inland storm drains that were discharging flow continuously during

dry weather. While it is possible that, in addition to the surveyed drains, multiple sources contributed to widespread human waste contamination along each creek transect, it is also entirely possible that these drains were contributing to coastal water quality degradation at the time of this study. This conclusion is based on the prevalence of human waste markers by qPCR along each transect. However, to more definitively determine the impact of inland drains on downstream coastal water quality, a better understanding would be needed of human waste marker decay and transport characteristics in the coastal creek environment. Still, this study shows the importance of examining urban storm drains as contributors of human waste contamination to surface waters, the complexity of determining how such waste enters drains, and the value of filling in host-specific DNA marker methodological gaps with profiling approaches that are widely available.

Multitiered source tracking has been used successfully before in southern California during dry weather (3, 32). Culture-independent microbial profiling has also been applied in microbial source tracking. For example, fingerprinting fecal sources on the basis of *E. coli* genotypes was used in source tracking (25) and PCR-TRFLP of eubacterial communities was used to distinguish environmental sands from deer fecal samples (9). TRFLP analysis was also the basis for development of *Bacteroides* gene markers for human and cow feces (2). We first tested the use of whole community TRFLP to discern fecal sources in an earlier methods-comparison study (18), but problems with recovering adequate DNA confounded the analysis. In another study, eubacterial primers did not offer sufficient sensitivity in clone library analysis of equine fecal contamination (43). Here, fecal sources were collected and analyzed, then their influence on aquatic communities was tested by16S PCR-TRFLP and multivariate analysis. The sensitivity afforded here, similarly to other studies using eubacterial primers in PCR-TRFLP (7, 11, 27-29) appeared adequate for ruling out the relative importance of other, various hosts' wastes. While PCR-TRFLP may not detect rare phylotypes, it is a highly reproducible and sensitive method for showing differences in bacterial communities along gradients. Grossly exemplifying the fact that TRFLP captures microbial community differences was that ocean water communities in this study were distinct, and communities along the transects appeared related based on their

relative position in the path of water flow. Thus TRFLP, and perhaps other similar DNA-based profiling methods, while not as data rich as clone library analysis or as specific as individual host-specific markers, appears useful as an intermediate tier of analysis in source tracking. Explanations for its performance in this context could include that, being whole community-based, it accounts for aggregated effects of the larger (i.e. chemical and biological) influences that discharged wastes have on indigenous microbial communities. Practically, PCR-TRFLP filled a gap in this study when either host-specific markers were not available or where their uncertain fate in the environment confounded their interpretation.

Flow from dry weather urban drains has been shown to carry FIB (34, 45) and, more specifically human waste, both by routine PCR of human *Bacteroides* gene marker and by RT-qPCR of human enterovirus (32). However, this study newly evaluated in-line, upstream origins of human waste, i.e. in an enclosed storm drain system upstream from the point where the pipe discharges into creek surface waters. This work points to interactions within subsurface urban infrastructure, i.e. between storm sewer pipes and other infrastructure in common trenches, as possible origins of human waste to creeks. However, the exact origins within the storm drain system were elusive, presumably because of the confounding effects of variable flow during sampling. Noble et al. (32) quantified the fluxes of cultivated FIB, qPCR-based Enterococcus, as well as RT-qPCR-based human enterovirus plus used routine PCR to show the presence of human *Bacteroides* gene markers emanating from drains and tributaries into Ballona Creek which drains to the Santa Monica Bay. In the latter study, the stability of signals in the creek and tributaries was impressive over the 6-hour daytime sampling period. In this study, we sampled in the morning over three successive days and quantified what appeared to be persistent qPCR-based evidence of human waste even though the signal magnitudes varied from day to day. Further, in each watershed, there was a consistent result that the highest concentrations of human waste DNA markers were associated with storm drain discharges. Thus, despite the fact that concentrations varied with possibly transient flow rates, it seems unequivocal that human waste contamination was evident along both transects away from storm drain discharges. However, we wonder to what degree, based on our study and those prior (i.e. (32) and (34)), storm drains discharge

human-specific waste that migrates all the way downstream into coastal zones during dry weather. Further, we wonder how many drains in urban environments are discharging human-associated waste. Ultimately, a full quantification of the phenomenon, better models and appropriate decay parameters for relating upstream to downstream concentrations, and learning the ultimate origins of infrastructure-associated contamination will be crucial for informing coastal water quality management in urban settings.

2.5 References

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TABLE 2-1: Physical characteristics of Mission Creek samples. DO = dissolved oxygen. Numbers in parentheses are standard error of the mean. N = 3 except where noted.

Site #	Description	DO (mg/L) Mean (SE)	Temp (˚C) Mean (SE)	Salinity (ppt) Mean (SE)	pH Mean (SE)
M1	Ocean surf	8.7 (0.3)	16.1 (0.4)	33.2 (0.3)	8.2 (0.1)
M2	Laguna lagoon	7.4 (0.6)	19.2 (0.4)	2.5 (1.4)	7.7 (0.2)
M3	Laguna channel	5.5 (0.2)	19.7 (0.0)	0.5 (0.0)	7.4 (0.0)
M4	Mission lagoon	10.0 (0.4)	18.9 (0.8)	6.4 (2.1)	8.3 (0.1)
M5	Montecito	5.2 (0.3)	18.4 (0.1)	0.5 (0.2)	7.8 (0.0)
M6	Haley-drain	5.8*	21.8*	0.0*	7.8 (0.0)
M7	Haley-creek	6.9 (0.5)	18.7 (0.2)	0.7 (0.0)	7.8 (0.0)
M8	OMC into MC	no data	no data	no data	7.9 (0.1)
M9	Westside drain	7.7 (0.2)	19.5 (0.5)	0.0 (0.0)	7.9 (0.0)

* unreplicated

TABLE 2-2: Physical characteristics of Arroyo Burro samples. DO = dissolved oxygen. Numbers in parentheses are standard error of the mean. N = 3.

Site #	Description	DO (mg/L) Mean (SE)	Temp (˚C) Mean (SE)	Salinity (ppt) Mean (SE)	pH Mean (SE)
A1	AB surf	8.4 (0.3)	17.7 (0.5)	30.6 (1.8)	8.2 (0.0)
A2	AB lagoon mouth	13.4 (0.6)	18.7 (0.5)	0.1 (0.0)	8.1 (0.0)
A3	above AB lagoon	8.5 (0.7)	18.4 (0.6)	1.1 (0.7)	7.9 (0.0)
A4	Mesa Creek/drain	8.8 (0.5)	17.6 (0.5)	0.0 (0.0)	8.2 (0.0)
A5	AB Creek @ Cliff Dr.	7.3 (0.5)	17.9 (0.6)	0.2 (0.2)	7.8 (0.0)
A6	AB below Las Positas Creek	7.8 (0.5)	17.9 (0.6)	0.0 (0.0)	7.9 (0.0)
A7	AB @ Hidden Valley Park	8.2 (0.4)	18.0 (0.3)	0.0 (0.0)	7.9 (0.0)
A8	AB downstream of Hope drain	no data	no data	no data	8.1 (0.0)
A9	Hope drain	no data	no data	no data	8.1 (0.0)
A10	Las Positas Creek @ Modoc	2.7 (1.3)	18.4 (0.8)	0.0 (0.0)	7.7 (0.2)

TABLE 2-3: Microbial measurements of Mission Creek samples. TC = total coliform, *E. coli* = *Escherichia coli*, Ent = enterococci (IDEXX), S = species richness, E = species evenness, H = Shannon diversity index (TRFLP-MDS). Numbers in parentheses are standard error of the mean. Superscripts indicate site(s) with significant difference (One-Way ANOVA with Dunnett's T3, α = 0.05). N = 3 except were noted.

Site #	TC (MPN/100mL) Mean (SE)	*E. coli* (MPN/100mL) Mean (SE)	Ent (MPN/100mL) Mean (SE)	S Mean (SE)	E Mean (SE)	H Mean (SE)
M1	1677 (1039)	138 (88)	31 (11)**	12.0 (0.6) M9,6	0.737 (0.025)	1.832 (0.091) M9,6
M2	>24196***	453 (255)	637 (430)	15.3 (0.7) M9	0.880 (0.006) M9	2.402 (0.041) M9
M3	21307 (1444)	377 (27)	318 (95)	18.0 (1.7) M9	0.861 (0.009) M9	2.482 (0.106) M9
M4	15733 (1597)**	2639 (1320)	1789 (1088)	15.0 (1.2) M9	0.803 (0.025)	2.168 (0.065) M9
M5	8754 (3279)**	3241 (1751)	437 (187)	13.7 (0.9) M9	0.882 (0.019)	2.300 (0.027) M9,6
M6	>24196***	3472 (1900)	11714 (4744)	20.3 (0.9) M9,7,1	0.875 (0.019)	2.632 (0.038) M9,7,5,1
M7	10403 (2005)	1769 (615)	485 (173)	13.0 (0.6) M9,6	0.869 (0.009)	2.225 (0.015) M9,6
M8	12997 (0)**	1297 (424)	574 (155)	23.3 (5.8)	0.840 (0.060)	2.617 (0.416)
M9	4407 (785)	484 (415)	122 (25)	36.0 (1.5) M7-1	0.948 (0.003) M3,2	3.396 (0.052) M7-1

**N =2; ***All values exceeded dilution range

TABLE 2-4: Microbial measurements of Arroyo Burro samples. TC = total coliform (IDEXX), *E. coli* = *Escherichia coli* (IDEXX), Ent = enterococci (IDEXX), S = species richness, E = species evenness, H = Shannon diversity index. Numbers in parentheses are standard error of the mean. Superscripts indicate site(s) with significant difference (One-Way ANOVA with Dunnett's T3, α = 0.05). N = 3 except were noted.

Site #	TC (MPN/100 mL) Mean (SE)	*E. coli* (MPN/100 mL) Mean (SE)	Ent (MPN/100 mL) Mean (SE)	S Mean (SE)	E Mean (SE)	H Mean (SE)
A1	2608 (2134)	184 (121)	27 (13) ^{A6,3}	14.0 (1.5) ^{A4}	0.743 (0.045)	1.964 (0.200)
A2	21307 (1444)	126 (53)	180 (47) ^{A8}	23.3 (0.7) ^{A7,6,5}	0.895 (0.019)	2.819 (0.087) ^{A6,5}
A3	19863*	203 (67)	256 (9) ^{A1}	19.3 (1.8)	0.844 (0.026)	2.496 (0.149)
A4	10568 (817)	399 (62)	1313 (354)	30.0 (1.5) ^{A7,6,5,1}	0.869 (0.011)	2.954 (0.078) ^{A6,5}
A5	24196 (0)**	515 (281)	277 (61) ^{A8}	12.7 (0.7) ^{A9,4,2}	0.785 (0.028)	1.989 (0.041) ^{A9,4,2}
A6	6293 (1458)	86 (17)	185 (9) ^{A1}	12.3 (0.9) ^{A9,4,2}	0.833 (0.003)	2.088 (0.058) ^{A9,4,2}
A7	10183 (7018)	320 (61)	274 (128) ^{A8}	16.3 (0.7) ^{A9,4,2}	0.779 (0.049)	2.170 (0.103)
A8	>24196***	981 (243)	2376 (214) ^{A7,5,2}	18.3 (0.9)	0.770 (0.022)	2.239 (0.096)
A9	198630*	9007 (2278)	25567 (15960)	25.0 (0.0) ^{A7,6,5}	0.870 (0.009)	2.800 (0.028) ^{A6,5}
A10	99315 (12675)**	15110 (7059)	9180 (4869)	22.3 (3.8)	0.859 (0.042)	2.653 (0.261)

*N =1; **N =2; ***All values exceeded dilution range

TABLE 2-5: SIMPER analysis on Mission Creek samples with sewage as a separate group. "Characteristic" or "key" peaks were considered to be those found in all members of the group.

Groups	Peaks found in all members of group	Cumulative % similarity for group
creek/drain/lagoon	none universal	0
ocean	93, 96, 341, 515	63.16
gull	216, 372, 583	100
raccoon	n/a	n/a
sewage	100, 202, 205, 224, 565, 591	59.91

Comparison of groups and % of dissimilarity and similarity

Comparison	% Dissimilarity	% Similarity
ocean & creek/drain/lagoon	86.33	13.67
ocean & gull	100	0
Creek/drain/lagoon & gull	94.23	5.77
ocean & raccoon	97.1	2.9
creek/drain/lagoon & raccoon	96.37	3.63
gull & raccoon	78.41	21.59
creek/drain/lagoon & sewage	77.32	22.68
gull & sewage	93.32	6.68
raccoon & sewage	98.44	1.56

TABLE 2-6: SIMPER analysis on Mission Creek samples, with Haley drain samples as a separate group. "Characteristic" or "key" peaks were considered to be those found in all members of the group.

Samples	Peaks found in all members of group	Cumulative % similarity for group
creek/drain/lagoon	none universal	0
ocean	93, 96, 341,515	63.16
gull	216, 372, 583	100
raccoon	n/a	n/a
sewage	100, 202, 205, 224, 565, 591	59.91
Haley drain	86, 88, 98, 138, 197, 202, 206, 358, 365, 367, 559, 564	88.11

Comparison of groups and % of dissimilarity and similarity

Comparison	% Dissimilarity	% Similarity
ocean & creek/drain/lagoon	85.13	14.87
ocean & Haley drain	94.79	5.21
creek/drain/lagoon & Haley drain	70.63	29.37
ocean & gull	100	0
creek/drain/lagoon & gull	93.57	6.43
Haley drain & gull	98.85	1.15
ocean & raccoon	97.1	2.9
creek/drain/lagoon & raccoon	96.42	3.58
Haley drain & raccoon	95.96	4.04
gull & raccoon	78.41	21.59
ocean & sewage	98.21	1.79
creek/drain/lagoon & sewage	77.91	22.09
Haley drain & sewage	73.22	26.78
gull & sewage	93.32	6.68
raccoon & sewage	98.44	1.56

TABLE 2-7: SIMPER analysis on Arroyo Burro samples with sewage as a separate group. "Characteristic" or "key" peaks were considered to be those found in all members of the group.

Samples	Peaks found in all members of group	Cumulative% similarity of group
creek/drain/lagoon	none universal	0
ocean	55, 57, 93, 96, 341, 515, 670	72.73
cat	n/a	n/a
dog	n/a	n/a
septage	n/a	n/a
sewage	100, 202, 205, 224, 565, 591	59.91

Comparison of groups and % of dissimilarity and similarity

Comparison	% Dissimilarity	% Similarity
ocean & creek/drain/lagoon	74.9	25.1
ocean & cat	87.78	12.22
creek/drain/lagoon & cat	94	6
ocean & dog	86.54	13.46
creek/drain/lagoon & dog	94.75	5.25
cat & dog	45.45	54.55
ocean & sewage	93.83	6.17
creek/drain/lagoon & sewage	77	23
cat & sewage	84.4	15.6
dog & sewage	81.33	18.67
ocean & septage	83.46	16.54
creek/drain/lagoon & septage	87.42	12.58
cat & septage	100	0
dog & septage	100	0
sewage & septage	94.23	5.77

TABLE 2-8: SIMPER analysis on Arroyo Burro samples, with Hope drain samples as a separate group. "Characteristic" or "key" peaks were considered to be those found in all members of the group.

Samples	Peaks found in all members of group	Cumulative % similarity
creek/drain/lagoon	none universal	0
ocean	55, 57, 93, 96, 341, 515, 670	72.73
cat	n/a	n/a
dog	n/a	n/a
septage	n/a	n/a
sewage	100, 202, 205, 224, 565, 591	59.91
Hope drain	55, 87, 96, 98, 139, 172, 202, 205, 206, 362, 366, 367, 565	86.67

Comparison of groups and % of dissimilarity and similarity

Comparison	% Dissimilarity	% Similarity
ocean & creek/drain/lagoon	74.31	25.69
ocean & Hope drain	79.59	20.41
creek/drain/lagoon & Hope drain	76.75	23.25
ocean & cat	87.78	12.22
creek/drain/lagoon & cat	95.58	4.42
Hope drain & cat	81.4	18.6
ocean & dog	86.54	13.46
creek/drain/lagoon & dog	95.97	4.03
Hope drain & dog	85.00	15.00
cat & dog	45.45	54.55
ocean & sewage	93.83	6.17
creek/drain/lagoon & sewage	77.45	22.55
Hope drain & sewage	73.34	26.66
cat & sewage	84.40	15.60
dog & sewage	81.33	18.67
ocean & septage	83.46	16.54
creek/drain/lagoon & septage	87.01	12.99
Hope drain & septage	90.7	9.3
cat & septage	100	0
dog & septage	100	0
sewage & septage	94.23	5.77

TABLE 2-9: FIB results for Phase 3 Haley drain tracking on August 15 – 17, 2006. TC = total coliform, *E. coli* = *Escherichia coli*, Ent = enterococci (IDEXX).

ID	Description	TC (MPN/100 mL)	*E. coli* (MPN/100 mL)	Ent (MPN/100 mL)
815-01	Haley drain diversion	>24196	12997	>24196
815-02	CDS unit	>24196	6488	>24196
815-03	manhole @ Haley & Chapala	>24196	>24196	>24196
815-04	manhole @ Chapala & Cota	>24196	933	4352
815-05	manhole @ Chapala & Ortega		not sampled	
815-06	drop inlet @ Chapala & Ortega	>24196	350	1904
815-07	sump 1 @ Paseo Nuevo	>24196	<10	428
815-08	sump 2 @ Paseo Nuevo	<10	<10	<10
815-09	sump 3 @ Paseo Nuevo	>24196	63	<10
815-10	Nordstrom's sump		not sampled	
816-01	Haley drain diversion	17250	2790	2110
816-02	CDS unit	61310	2260	2880
816-03	manhole @ Haley & Chapala	64880	6500	6440
816-04	manhole @ Chapala & Cota	>24196	175	1607
816-05	manhole @ Chapala & Ortega	>24196	19863	>24196
816-06	drop inlet @ Chapala & Ortega	>24196	6867	24196
816-07	sump 1 @ Paseo Nuevo	>24196	<10	120
816-08	sump 2 @ Paseo Nuevo	10	<10	<10
816-09	sump 3 @ Paseo Nuevo	>24196	41	<10
816-10	Nordstrom's sump	2064	10	20
817-01	Haley drain diversion	64880	30760	3450
817-02	CDS unit	98040	46110	4880
817-03	manhole @ Haley & Chapala	61310	43520	3270
817-04	manhole @ Chapala & Cota	6910	<100	520
817-05	manhole @ Chapala & Ortega	>24196	75	689
817-06	drop inlet @ Chapala & Ortega	43520	630	2920
817-07	sump 1 @ Paseo Nuevo	>24196	<10	74
817-08	sump 2 @ Paseo Nuevo	16	<1	<1
817-09	sump 3 @ Paseo Nuevo	>241960	100	<100
817-10	Nordstrom's sump	>2419.6	>2419.6	>2419.6

TABLE 2-10: FIB results for Phase 3 Hope drain tracking on September 5 - 7, 2006. TC = total coliform, *E. coli* = *Escherichia coli*, Ent = enterococci (IDEXX).

ID	Description	TC (MPN/100 mL)	*E. coli* (MPN/100 mL)	Ent (MPN/100 mL)
905-01	Hope drain diversion	>24196	2755	6867
905-02	drop inlet near Starbucks	>24196	5172	2481
905-03	side drain near Red Robin	24196	<10	6131
905-04	manhole @ Ritz Camera & Vons	>24196	>24196	14136
905-05	manhole near Cingular	>24196	>24196	24196
905-06	drop inlet near La Salsa	>24196	>24196	>24196
905-07	bus stop near Spectrum Gym	>24196	17329	>24196
906-01	Hope drain diversion (8:10)	<10	<10	10
906-02	drop inlet near Starbucks	>24196	17329	5794
906-03	side drain near Red Robin	11199	2987	301
906-04	manhole @ Ritz Camera & Vons	>241960	173290	20980
906-05	manhole near Cingular	>241960	129970	19350
906-06	drop inlet near La Salsa	>241960	24810	14210
906-07	bus stop near Spectrum Gym	>241960	310	<100
906-08	Hope drain diversion (10:00)	>24196	>24196	19863
907-01	Hope drain diversion	>24196	>24196	24196
907-02	drop inlet near Starbucks	>24196	>24196	19863
907-03	side drain near Red Robin	>24196	>24196	>24196
907-04	manhole @ Ritz Camera & Vons	>241960	32550	17850
907-05	manhole near Cingular	>241960	68670	17230
907-06	drop inlet near La Salsa	>241960	9320	13330
907-07	bus stop near Spectrum Gym	>24196	3448	5794

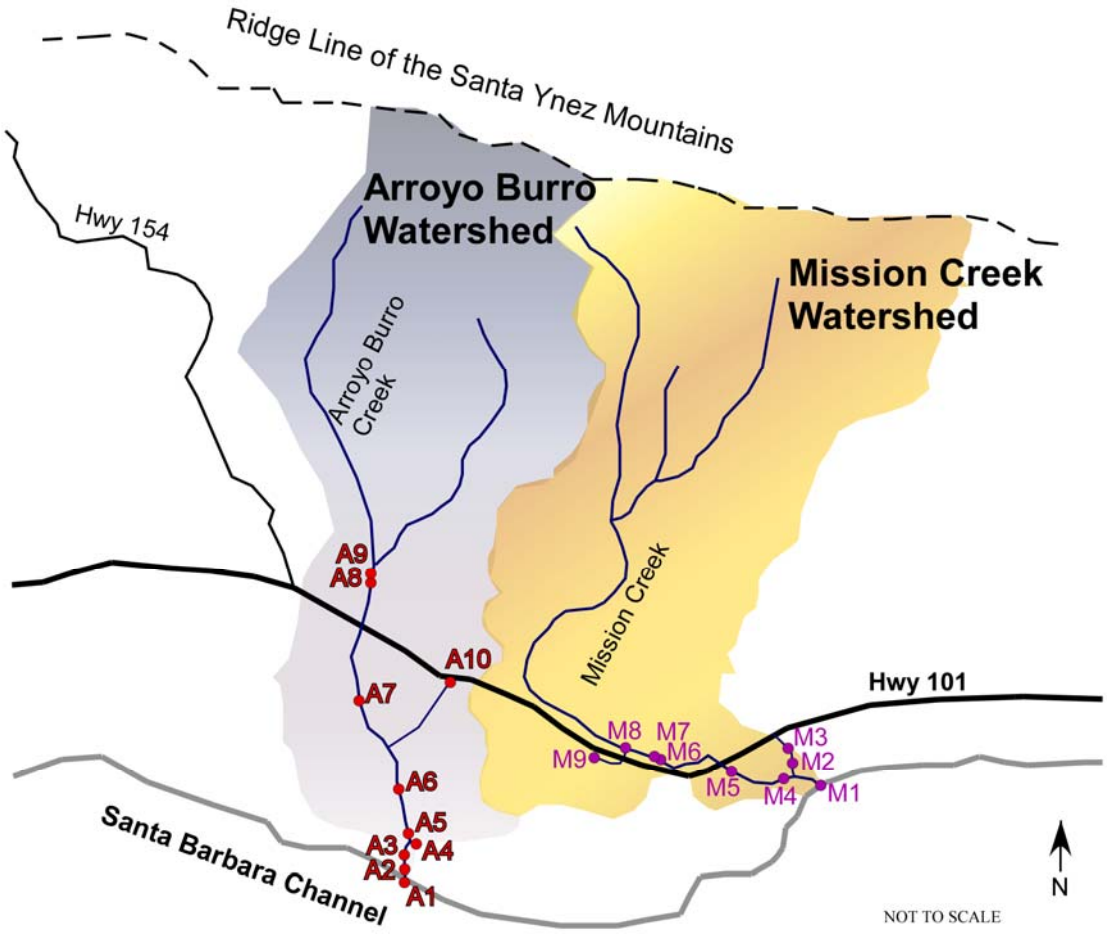

Figure 2-1: Map of the two watersheds in Santa Barbara, California, targeted in this study (Mission Creek and Arroyo Burro), and the primary sampling locations in each.

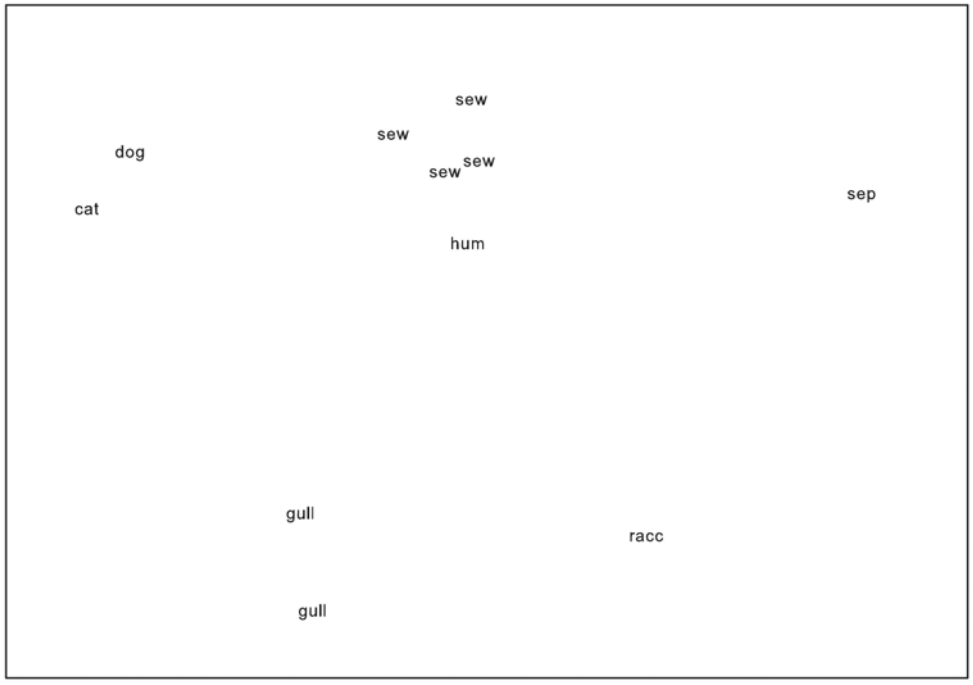

FIGURE 2-2: MDS plot of all fecal sources sampled in this study: sewage (sew), human (hum), septage (sep), gull, raccoon (racc), cat, and dog (presence/absence, stress = 0.05).

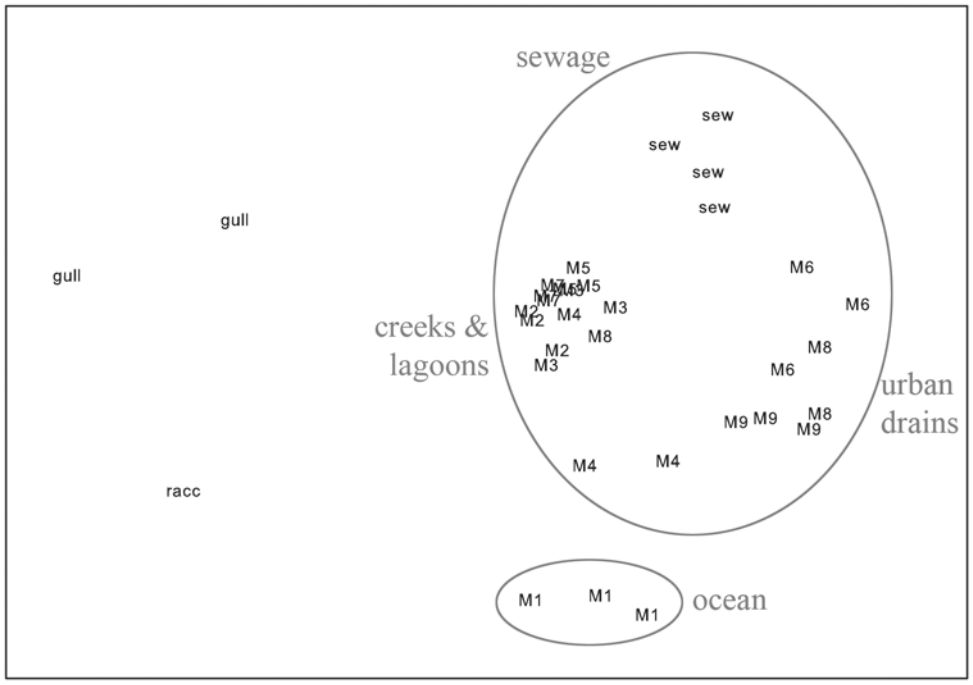

FIGURE 2-3: MDS plot of the Mission Creek samples, sewage, gull, and raccoon fecal sources (presence/absence, stress = 0.13). The sewage/creeks/lagoons/urban drains group, ocean group, gull samples, and raccoon were all statistically different from each other (ANOSIM, P > 0.001).

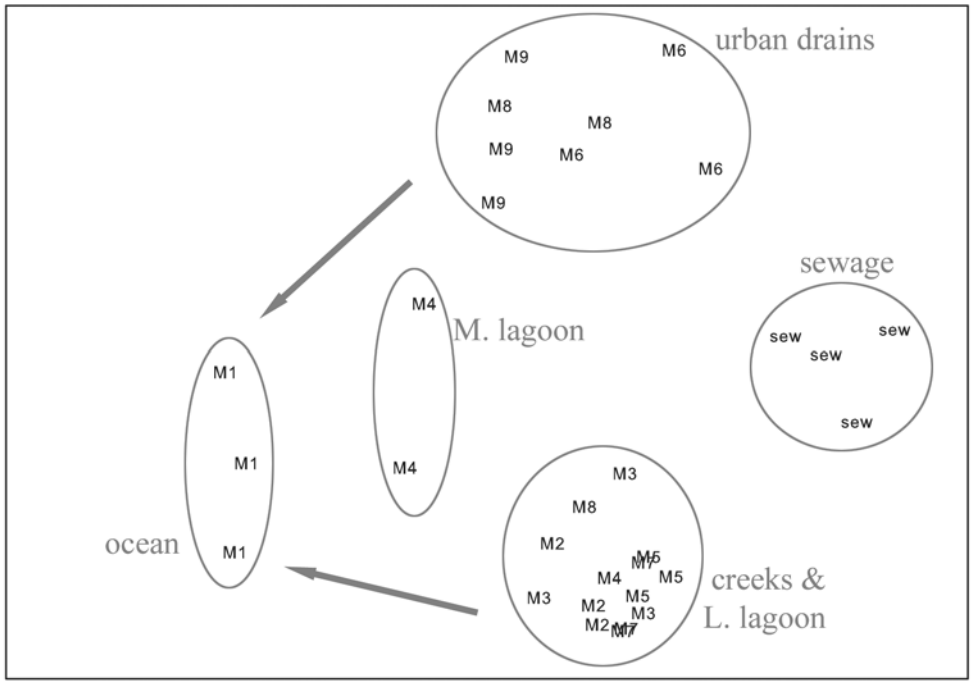

FIGURE 2-4: MDS plot of the Mission Creek samples and sewage (presence/absence, stress = 0.14). Arrows indicate direction of flow from upstream to downstream in system.

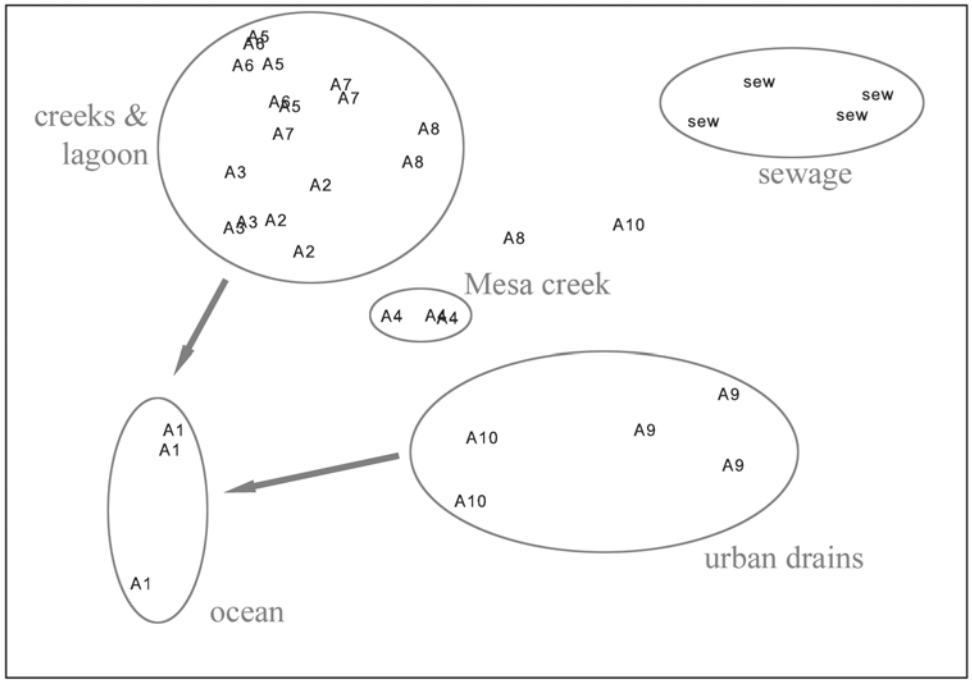

FIGURE 2-5: MDS plot of the Arroyo Burro samples and sewage (presence/absence, stress = 0.14). Arrows indicate direction of flow from upstream to downstream in system.

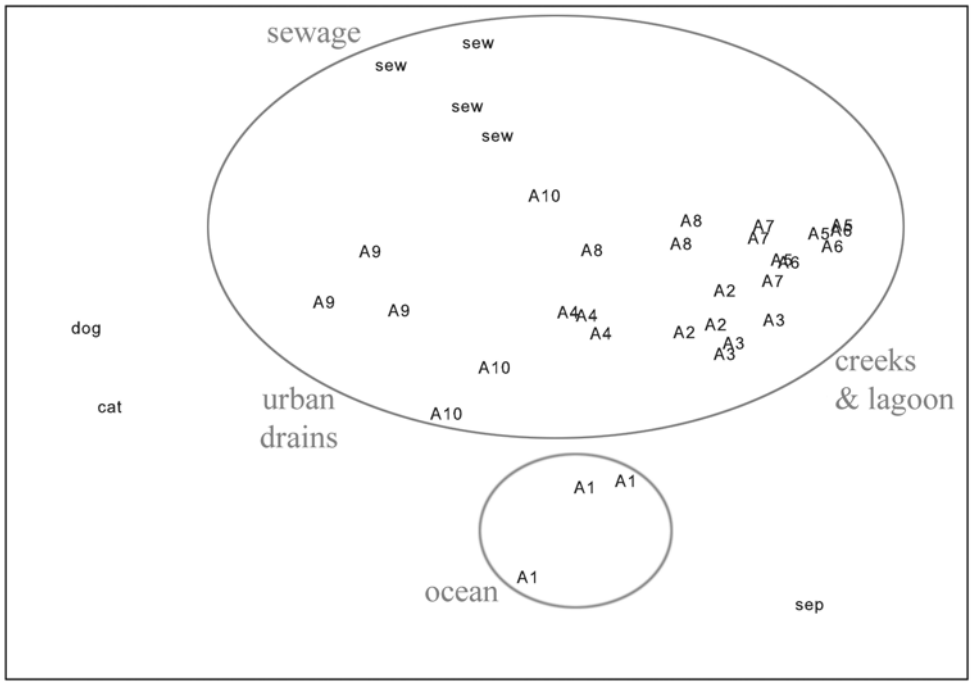

FIGURE 2-6: MDS plot of the Arroyo Burro samples, sewage, septage, cat and dog fecal sources (presence/absence, stress = 0.15). The sewage/creeks/lagoon/urban drains group, ocean group, dog, cat, and septage were all statistically different from each other (ANOSIM, P < 0.001).

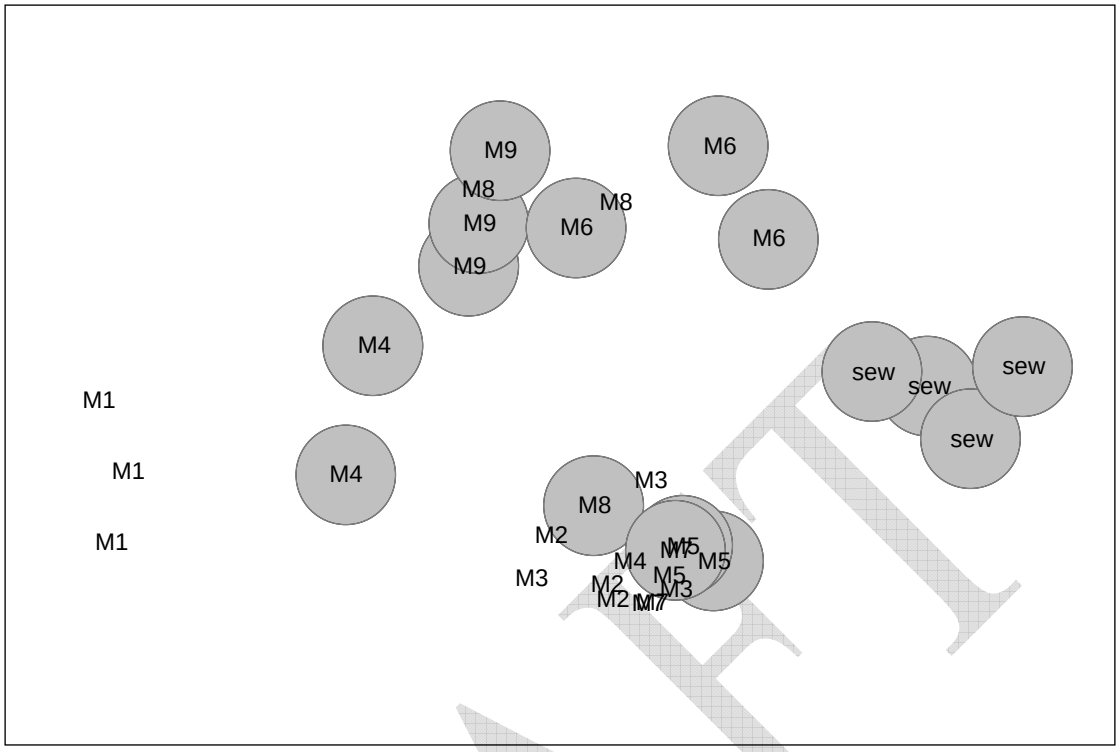

FIGURE 2-7: MDS plot of Mission Creek samples and sewage (same as Figure 4) with bubble overlay for peak #202.

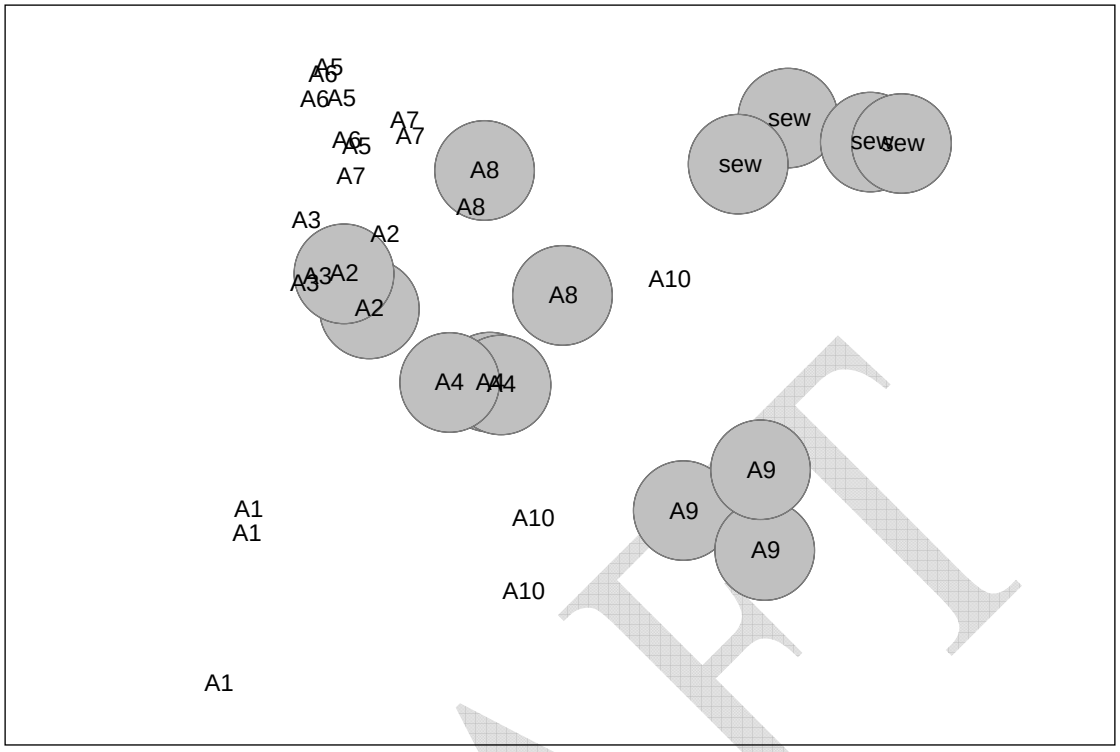

FIGURE 2-8: MDS plot of Arroyo Burro samples and sewage (same as Figure 5) with bubble overlay for peak #202.

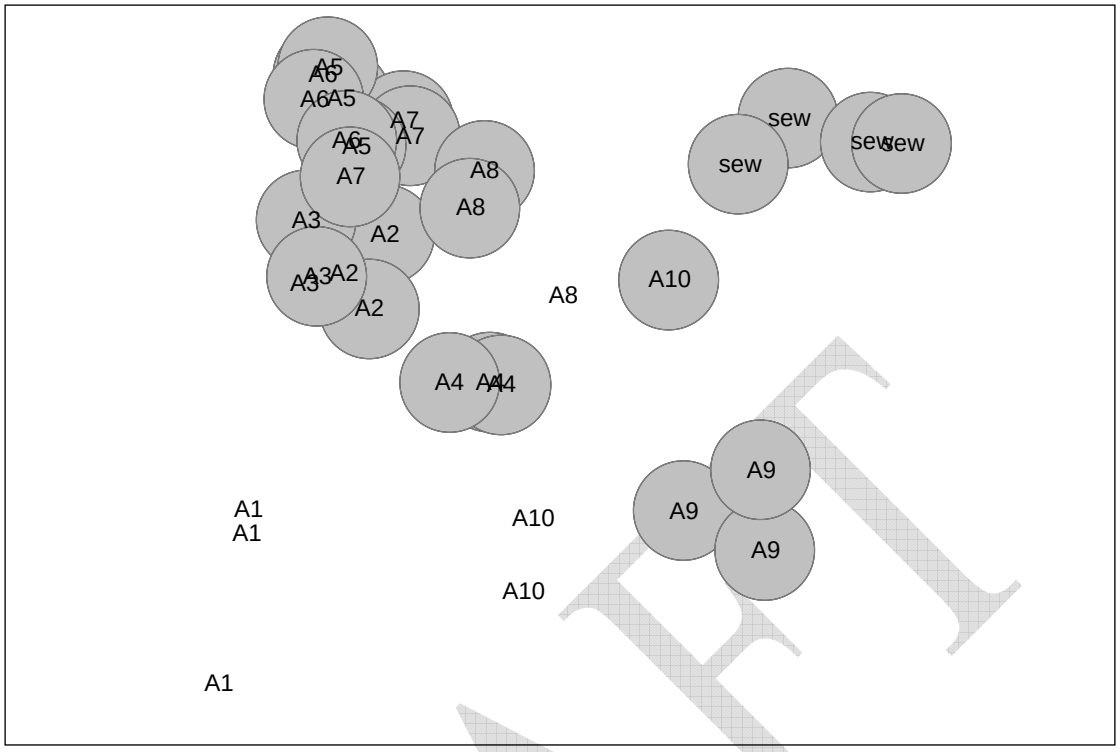

FIGURE 2-9: MDS plot of Arroyo Burro samples and sewage (same as Figure 5) with bubble overlay for peak #205.

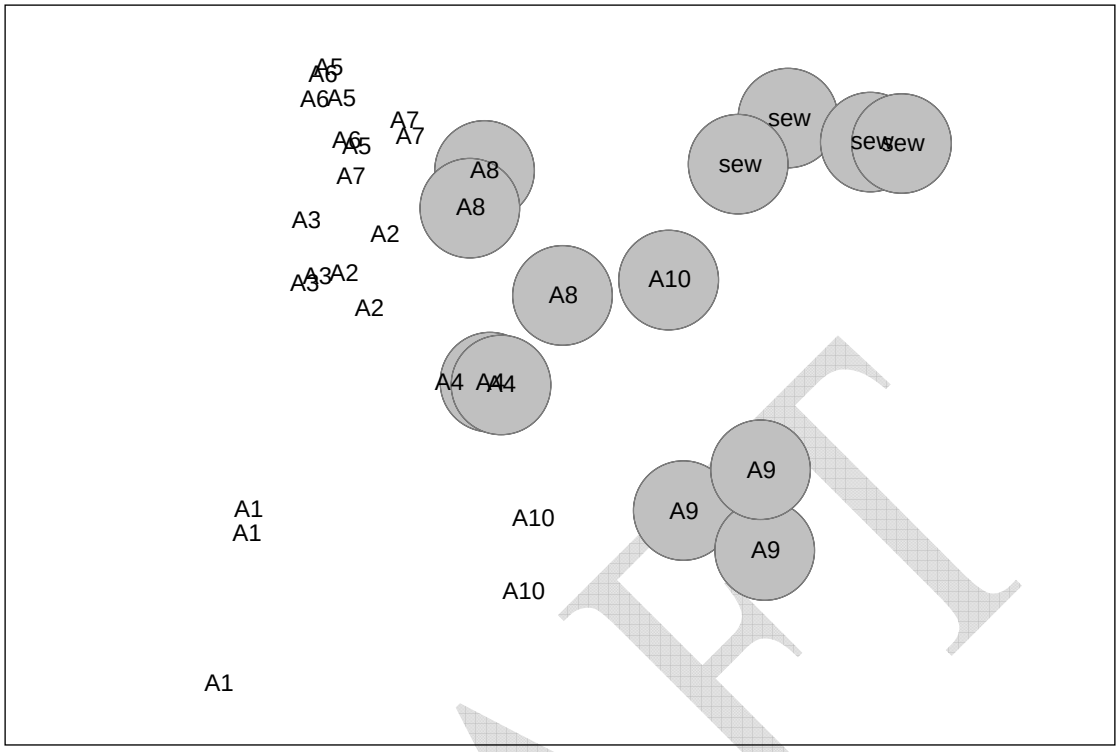

FIGURE 2-10: MDS plot of Arroyo Burro samples and sewage (same as Figure 5) with bubble overlay for peak #565.

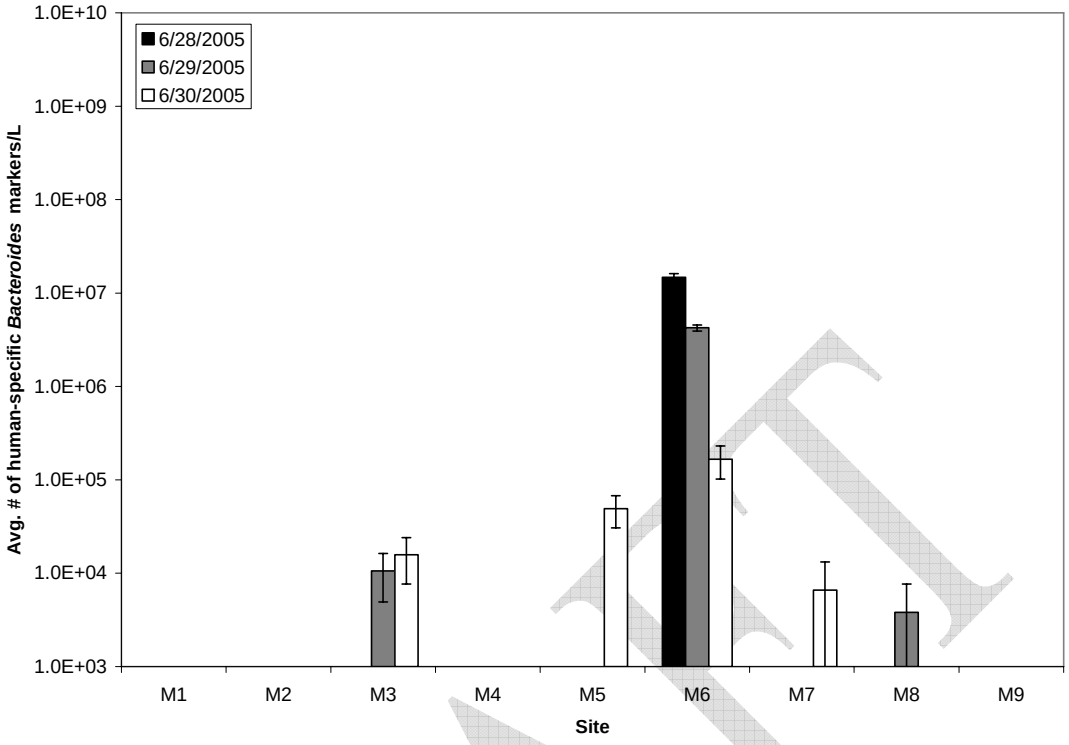

FIGURE 2-11: Human-specific *Bacteroides* qPCR results for Mission Creek samples, expressed as the average number of human-specific *Bacteroides* markers per liter. Error bars represent the standard error of the analytical replicates for each sample. Site M6 (Haley drain) was statistically different from the other sites (One-Way ANOVA with Dunnett's T3, $\alpha = 0.05$).

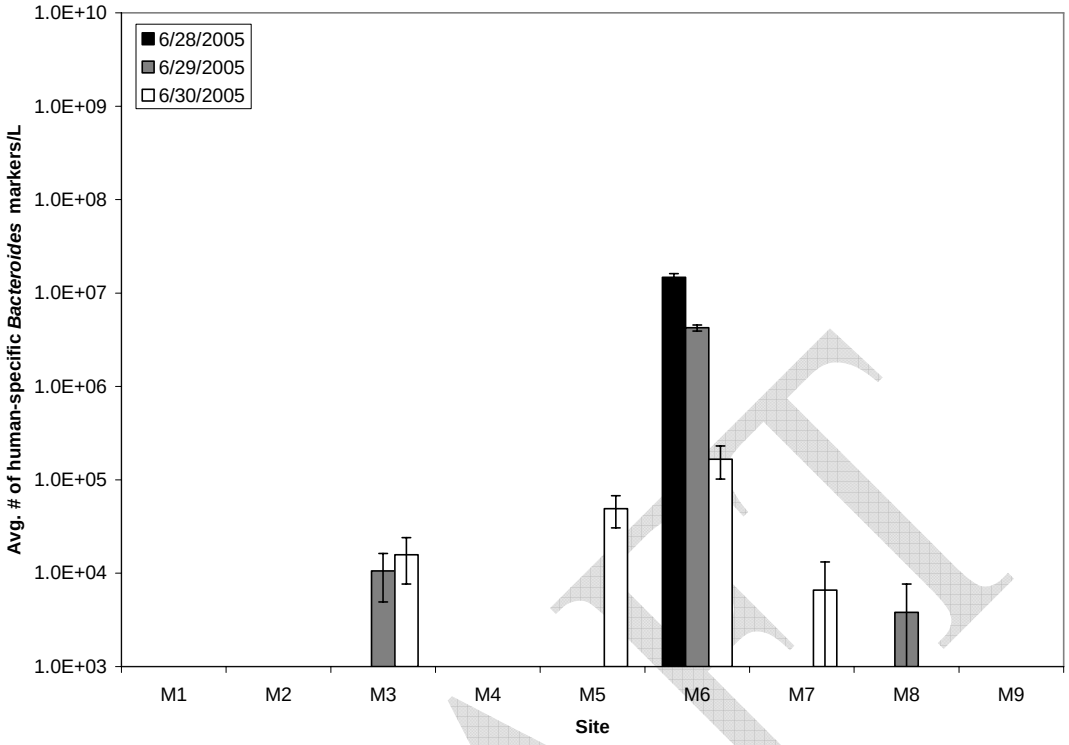

FIGURE 2-12: Human-specific *Bacteroides* qPCR results for the Arroyo Burro samples, expressed as the average number of human-specific *Bacteroides* markers per liter. Error bars represent the standard error of the analytical replicates for each sample. No sites were statistically different from the other sites (One-Way ANOVA with Dunnett's T3, $\alpha = 0.05$).

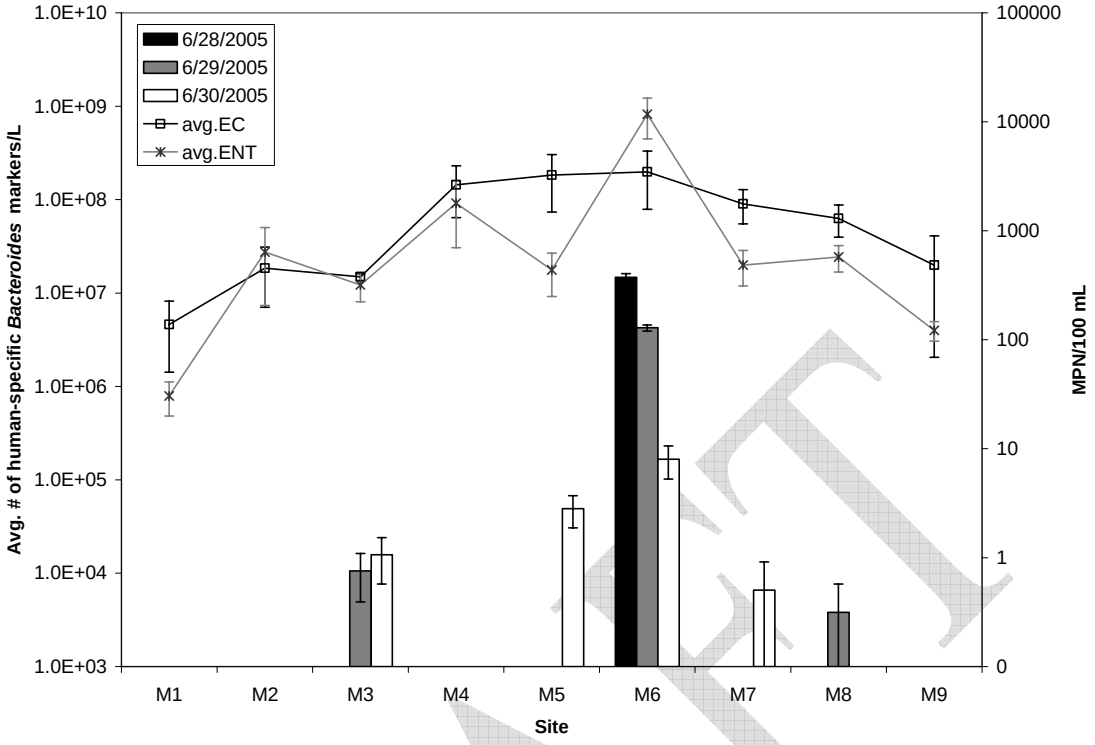

FIGURE 2-13: Human-specific *Bacteroides* (displayed as bars), *E. coli* and enterococci (displayed as lines) results for Mission Creek samples. For the *Bacteroides* results, error bars represent the standard error of the analytical replicates. *E. coli* and enterococci results are expressed as the average (and standard error) of the three consecutive snapshot sampling days. Similar patterns suggest a log-log relationship.

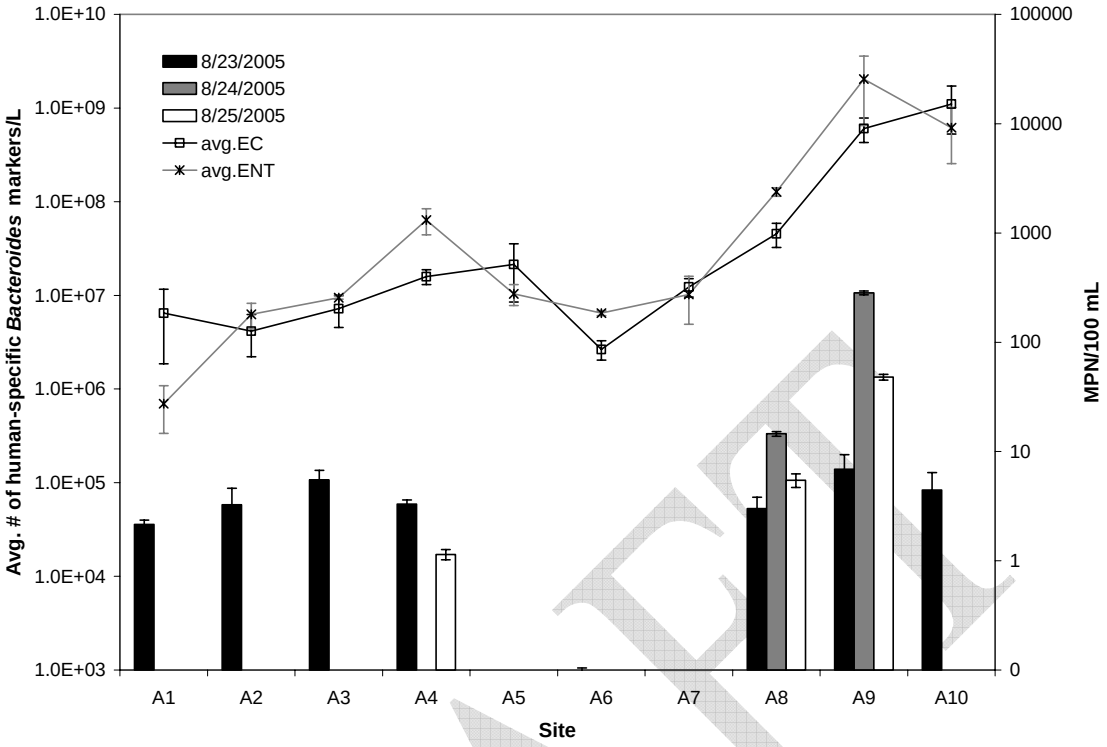

FIGURE 2-14: Human-specific *Bacteroides* (displayed as bars), *E. coli* and enterococci (displayed as lines) results for Arroyo Burro samples. For the *Bacteroides* results, error bars represent the standard error of the analytical replicates. *E. coli* and enterococci results are expressed as the average (and standard error) of the three consecutive snapshot sampling days. Similar patterns suggest a log-log relationship.

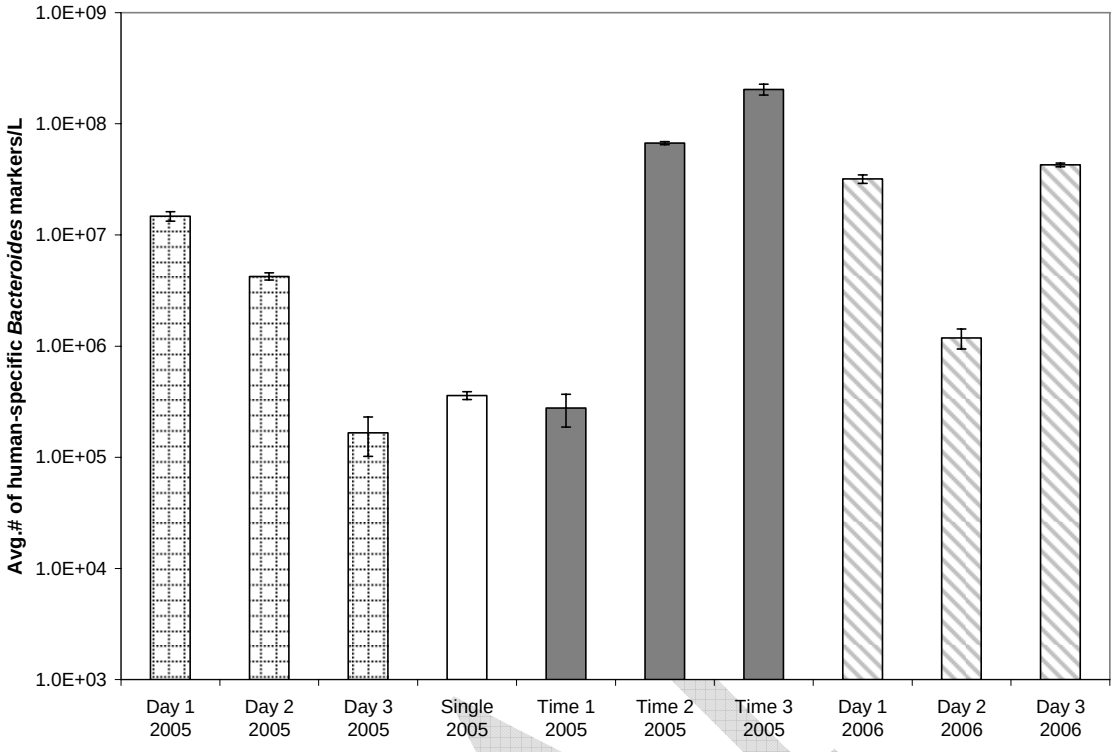

FIGURE 2-15: Human-specific *Bacteroides* qPCR results for all sampling events at Haley drain (M6). Error bars represent the standard error of the analytical replicates for each sample. The average number of markers varied from 1.7E+05 to 2.0E+08 markers/L (= 0.002 to 2.6% of sewage) overall, and from 2.8E+05 to 2.0E+08 markers/L (= 0.004 to 2.6% of sewage) in a single day (shown in darker gray bars, 2005, Time 1 = 8:30, Time 2 = 11:55, Time 3 = 14:25).

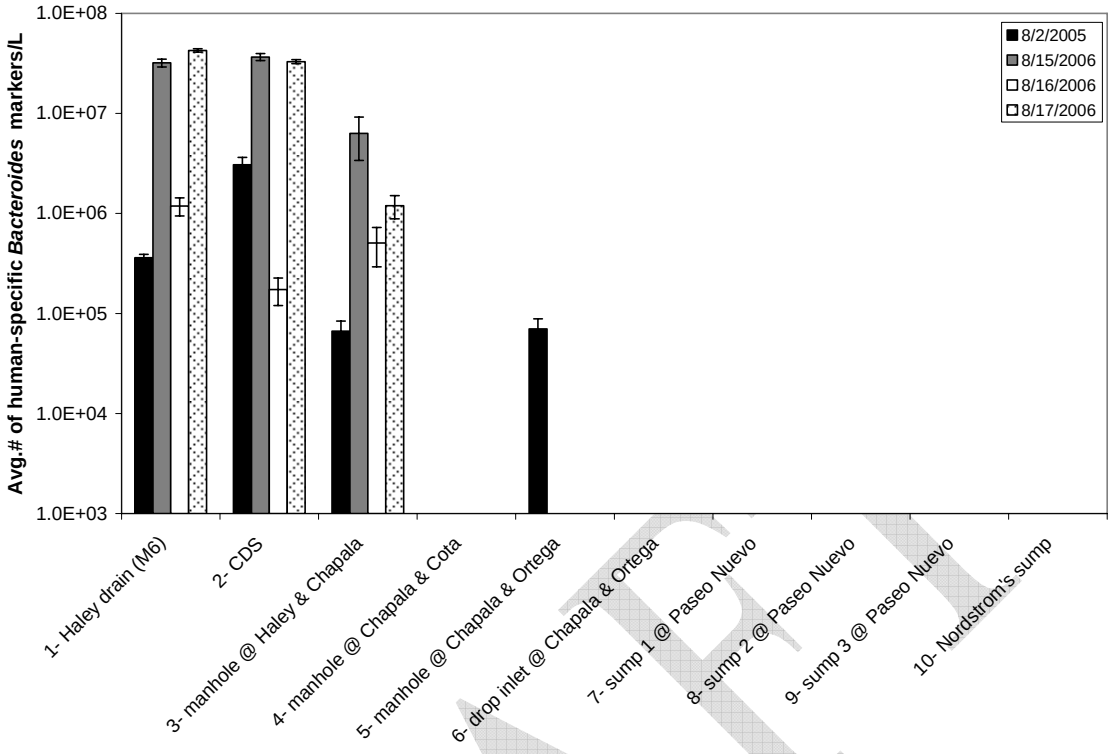

FIGURE 2-16: Haley drain tracking human-specific *Bacteroides* qPCR results for the one-day 2005 sampling event, and the three-day 2006 sampling events. For 2005, sites #4 and 6-10 were not sampled. For 2006, sites #5 and 10 were not sampled on the first day (8/15/2006). Otherwise, absence of data indicates either no target amplification or amplification was below the limit of quantification. Error bars represent the standard error of the analytical replicates for each sample.

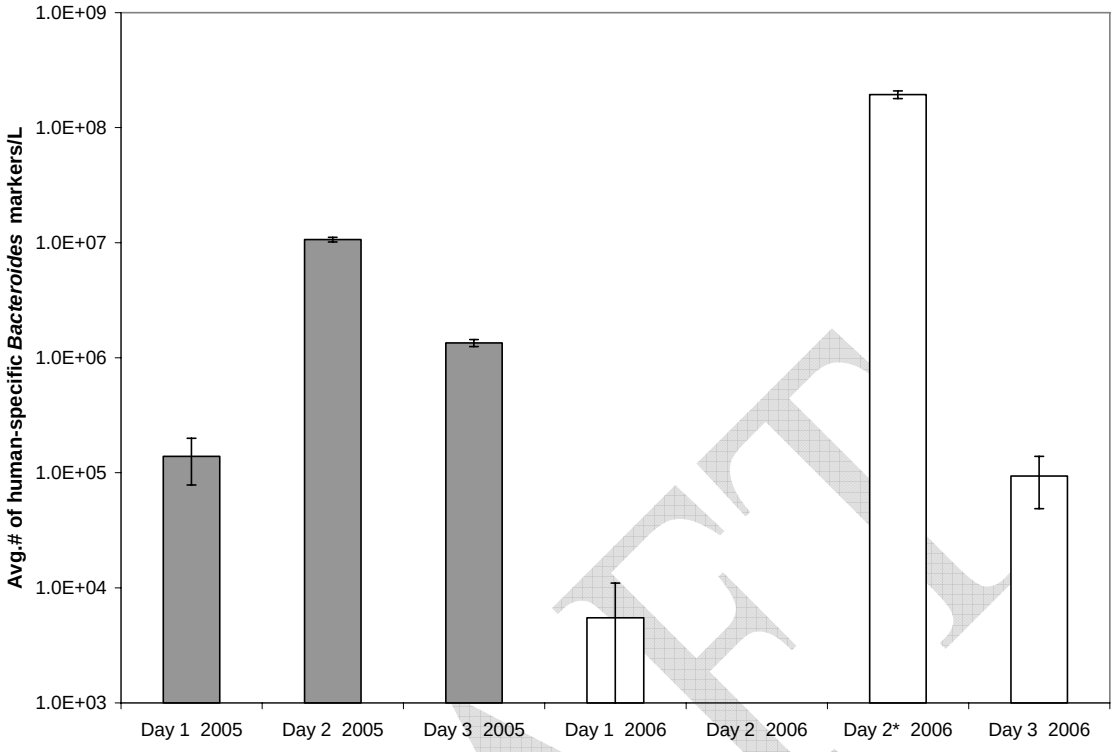

FIGURE 2-17: Human-specific *Bacteroides* qPCR results for all sampling events at Hope drain (A9). Error bars represent the standard error of the analytical replicates for each sample. Hope drain was sampled twice on Day 2 in 2006 due to a noticeable difference in water flow and color (Time 1 = 08:10, Time 2 = 10:00). The average number of markers varied from below the limit of quantification to 1.9E+08 markers/L (= < 0.0001 to 2.5% of sewage) in less than 2 hours.

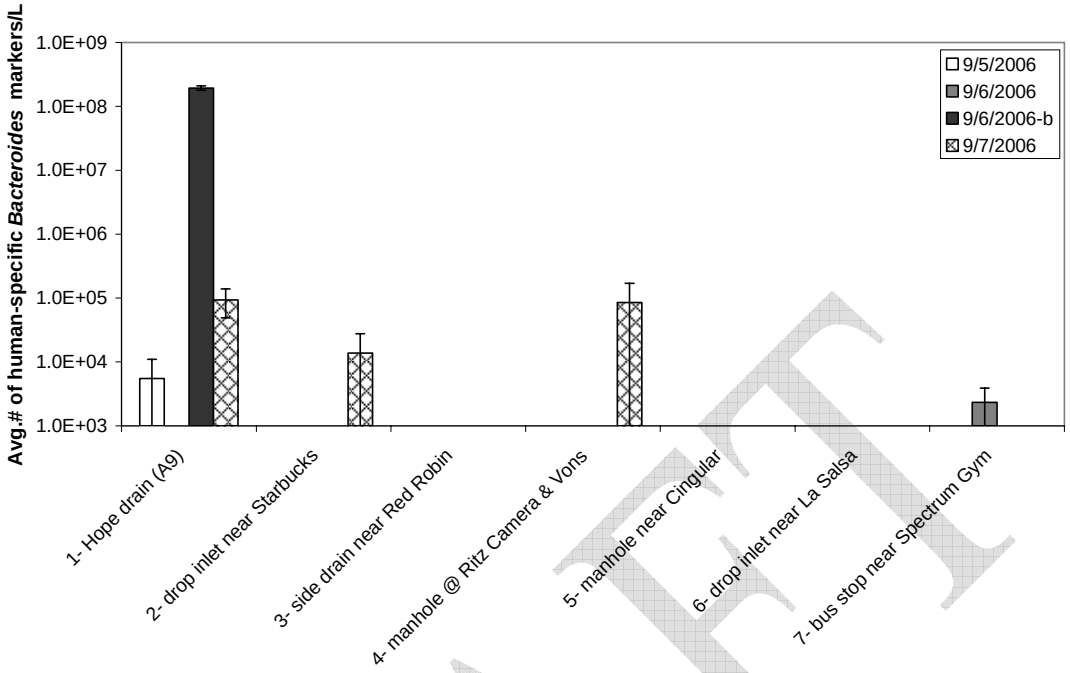

FIGURE 2-18: Hope drain tracking human-specific *Bacteroides* qPCR results for the three-day 2006 sampling events. Hope drain (A9) was sampled twice on 9/6/2006 due to a noticeable difference in water flow and color (Time 1 = 08:10, Time 2 = 10:00). Absence of data indicates either no target amplification or amplification was below the limit of quantification. Error bars represent the standard error of the analytical replicates for each sample.

Chapter 3: Fate and Transport of Human Waste Downstream of a Storm Drain Discharge (Phase IIB and the Phase II Dispersion study, plus the Phase III Haley Sediment study)

3.1 Introduction

Fecal indicator concentrations in streams vary with varying inputs related to watershed urbanization, season and with storm flow (8). Concentrations may also vary because of microbial growth or attenuation of populations *in situ.* A general expression of fate and transport of a biological analyte, C, as it migrates with distance "x" along a surface water body (7), is:

$$\frac{\partial C}{\partial t} = -v\frac{\partial C}{\partial x} + D\frac{\partial^2 C}{\partial x^2} - kC_{x,t} \qquad (1)$$

where, irrespective of specific units:

C = concentration (M/L^3)

v = velocity (L/T)

t = time (T)

D = dispersion coefficient (L^2/T)

k = first order loss coefficient (1/T); and $k = k_d + \frac{V_s}{H} f$ (2)

where k_d is the biological decay coefficient, V_s is the settling coefficient for particle-associated organisms, H is the depth, and f is the fraction that are particle-associated.

As implied, fecal indicator bacteria can be attenuated or lost by sedimentation and decay (3). Decay rates of indicator bacteria in the environment vary with waste type, bacterial strain (4) and environmental factors (1). DNA-based markers of human waste and other waste offer the possibility of more rapidly and specifically diagnosing water quality, but they may also be labile and thus only useful for source identification in fresh

wastes. New findings that some *Bacteroides* sp. can growth in low oxygen conditions (2) and aerobically (9) test prior assumptions about *Bacteroides*-based DNA markers only being present in fresh waste, and imply that such tracers may attenuate slowly and perhaps amplify (9), in the environment. But currently, little is known regarding DNA-based marker fate in the environment (6). Because it is neither practical nor affordable to assay pathogens directly, tracers as close to pathogen behavior as possible and as specific to waste sources as possible are used for finding sources and understanding their migration patterns. But to understand if inland urban drainage can degrade coastal water quality and threaten public health, the behavior of DNA-based tracers of human waste, as well as conventional indicator bacteria, need to be better understood. Rather than lumping loss processes into a first order decay coefficient, accounting for individual processes such as decay, sedimentation, and dispersion is preferable (3, 7). Also, given that decay coefficients will vary with light, temperature, DO, pH and other field variables, decay coefficients must be assessed under field-relevant environmental conditions (1).

In a prior study (Chapter 2) of a human waste-contaminated creek, i.e. lower Mission Creek in Santa Barbara, CA, variations in concentrations of human waste-associated gene markers were observed both up and downstream of a storm drain that discharged into a creek. Because dry weather drainage carrying human waste could be delivered to the coastal ocean and thus affect recreational swimmers, this study was undertaken to further examine the transport and fate characteristics of fecal contamination in the subject urban coastal creek over a very short reach.

3.2 Materials and Methods

3.1.1 General approach

The overall approach in this study involved both field time course sampling away from a point source of human waste contamination (Haley Drain, Chapter 2) on Mission Creek and data analysis to infer the potential fate of microbial water contamination downstream. Also, field sampling was performed upstream of the drain discharge, within the drain system, to assess in-line characteristics of indicator bacterial concentrations

relative to human waste markers. To facilitate the field microbial data interpretation in the context of the field setting, a dispersion coefficient (D, eq. 1) was measured using a dye study over the field sampling creek reach.

3.1.2 Field time course sampling

For the field sampling study, water samples were taken on 08/04/05 during 3 different time intervals (7:45 – 9:00; 11:15 – 12:00; 13:45 – 14:45) at the Haley Drain outfall and at 3 locations in Mission Creek: Gutierrez, Downstream of Haley drain and Upstream of Haley drain. Flow rates of Mission Creek were measured by the stage recorder in Mission Creek at Montecito St. (constant at 0.013 $m^3 s^{-1}$). Additional flow measurements were made manually in Mission Creek at the upstream and downstream from Haley drain sampling locations. This was performed by measuring water velocities and water depth at 10-15 cm intervals along the width of the creek. The total flow rate was obtained by the sum of the flow rates (= velocity x cross-sectional area) for each interval. Flow rates of the drain outfall were also measured. Moreover on 08/02/05 water samples were taken at 4 locations in the drain system (9:35 – 11:00): Haley drain, CDS unit, HC and CO. Flow rates were not measured when these samples were collected, except for the drain. Sampling upstream of the drain within the storm drain system, in addition to downstream of the drain in the creek environment, allowed for comparatively assessing relationships between indicator organism concentrations and HGM concentrations across these two, possibly differently selective, environments. Samples were subjected to analysis of fecal indicator bacteria and DNA was extracted for analysis of HGM (see Chapter 2 Methods).

3.1.3 Dye study

A dye study was performed to determine the average water velocity and longitudinal dispersion coefficient for Mission Creek between the location downstream from the drain and Gutierrez. Approximately 0.20 L Rhodamine WT was diluted in approx. 15L of Creek water, and released downstream of Haley Drain. The Creek water

was immediately mixed to equalize cross-sectional dye concentrations. Water samples (20 mL) were collected downstream at Gutierrez, at 3 locations (left bank, middle, right bank), with 2-3 minutes intervals between sampling. Fluorescence was measured in real-time using a field fluorometer, in order to optimize sampling timing. All samples were stored at 4 ºC in the dark until determination of the fluorescence in the lab (after 3 days). At the same time, a calibration curve was made, using Creek water as diluent. The distance between dye release and dye sampling was 525 ft (160 m).

3.1.4 Sediment study

A sediment study was performed to determine if bacteriological and DNA-based markers for human waste have been stored in the sediments near Haley drain, and to assess the concentrations of fecal indicator bacteria and indicator DNA in sediments as compared to overlying water. Five sites were sampled: a far downstream location (Guttierrez), close downstream (just downstream of the drain), near the drain itself, upstream of the drain past the bridge, and a far upstream location (Cota).

At each sampling location, water samples were collected as described in previous chapters. Next, using 60 mL syringe corers, 3 to 5 sediment cores were taken at a depth of 2 to 3 cm per core and dispensed in 50 mL Falcon tubes for storage and transport. In the lab, any stones or particles larger than 3 mm were removed and approximately 1 g was utilized for % moisture determination as done in Phase I. An additional 1 g was archived for DNA extraction and stored at -20C. For FIB analysis of the sediments, 7 g was diluted with 40 mL of sterile Nanopure water, vortexed for 2 minutes, left undisturbed for 10 minutes to settle, and 5 mL of the supernatant was utilized in each assay.

IDEXX processing, DNA extraction, and human-specific *Bacteroides* qPCR were all carried out as before (see Chapter 2).

3.3 Results

3.3.1 Significance of FIB Input from Haley Drain into Mission Creek

Fig. 3-1 shows the FIB load of Haley drain into Mission Creek, measured during 3 time points on 08/04/05. The total bar height indicates the theoretical FIB load downstream of Haley drain, and the contributions of upstream (UP) and the drain (Drain) are separately indicated in the bar. The contribution of the drain to the total load of FIB in the creek downstream depended on the FIB and increased from *E. coli* (5-24%) < enterococci (12-58%) < human-specific *Bacteroides* (31-93%).

The high contribution of human-specific *Bacteroides* from the drain indicates that the drain is a significant source of human fecal pollution in Mission Creek. This also means that only measuring fluxes of traditional FIB (*E. coli* and enterococci) will underestimate the input of human fecal material in the creek.

A pattern of increased contribution of the drain to the FIB load in Mission Creek later during the day was also observed (except for *E. coli*, in this case a small decrease was observed in the afternoon). At each time point, the relative dominance of the human-specific *Bacteroides* input was always maintained.

3.3.2 Fate of FIB released into the Creek

On 08/04/05, all FIB concentrations were measured downstream from Haley drain at 2 locations (Down and GUT), again during 3 time intervals. The following assumptions were made:

1. Location DOWN (20m from drain outfall): The FIB concentrations will be the same as the ones calculated using a mass balance of upstream and drain data. Even at a decay rate of 2 d^{-1} (high estimate), only a 0.2% decrease in FIB concentrations is expected.
2. Location GUT (180m from drain outfall): The change in FIB concentrations between DOWN and GUT can be used to estimate decay rates (k_d) of the FIB, using the analytical solution of the 1-D advection-diffusion model for a continuous source: $C=Co*\exp(-k_d t)$. Using this model implies that longitudinal

dispersion does not significantly affect the concentrations downstream of a continuous source. For the concentration at DOWN, we used the theoretical values as calculated above, since the measured concentrations may be biased by incomplete mixing.

At DOWN, the measured FIB concentrations were mostly higher than the calculated concentrations (Fig. 3-2). For *E. coli* and enterococci, respectively, the observed concentrations were 2-5 and 6-16 times higher, than the calculated concentrations. This agrees with the previous observation that the drain outfall contributes more of the creek load for enterococci than for *E. coli*. However, this reasoning does not work for the human-specific *Bacteroides*. In that case, the disagreement between the measured and calculated concentrations was more extreme, and the ratios of measured/calculated concentrations ranged from 0.4 – 52. The latter extremely high ratio (52) was caused by the measurement of an unusually low concentration in the drain (at 8:30). If we would assume that the concentration of human-specific *Bacteroides* had increased to the average levels at the time the sample downstream was taken (at 9:00), the agreement between measured and calculated concentrations would be more favorable (ration of ~1.6). Although we can't test this hypothesis, it indicates that the temporal variations in bacterial concentrations do occur, and that they could have a major influence on the calculations of mass balances in the creek.

Overall, the disagreement between the measured and calculated FIB concentrations at this location indicates that mixing in the cross-sectional area was probably not complete at that sampling point. The latter is in accordance with rule-of-thumb calculations of mixing in rivers, indicating it may even take 200m before complete mixing in the cross-sectional area occurs after an input located along the river bank.

The FIB concentrations at DOWN and GUT, and corresponding decay rates are shown in Fig. 3-3. Both increasing and decreasing FIB concentrations are found from DOWN to GUT. The decay rates are very variable and not within the expected range (e.g. 0.1 - 2 d^{-1}), and some are even negative. Losses due to sedimentation are expected to be in the

range of 0.3 d^{-1}, so they cannot account for the large k_d values that are observed in some cases here.

The following factors may contribute to the unrealistically high and variable decay rates:

- Incomplete cross-sectional mixing at GUT
- Temporal variation in drain load
- Temporal variation in load upstream of drain
- Decoupling of time between DOWN and GUT samples (i.e. advection will cause DOWN sample to reach GUT 3 hours later, but we related DOWN sample with GUT 1 hour earlier).

Even when accounting for the time decoupling (i.e. relating DOWN concentrations with GUT concentrations ~2 hours later) this didn't improve the variability or magnitude of the decay rates.

In general, the data indicated that the concentration decrease from DOWN to GUT depends on the FIB. Especially for human-specific *Bacteroides*, concentrations decreased more rapidly than for *E. coli* and *Enterococcus*. However, the variability/insufficient mixing in the creek prevent accurate prediction of decay rates.

3.3.3 Drain Tracking

Our data suggested the drain is a significant source of human fecal pollution to the creek under dry weather conditions (Fig. 3-1). FIB concentrations in the drain were determined at several locations upstream of the drain outfall (Fig. 3-4), in order to try to discern the source of the human fecal pollution in the drain. A pronounced maximum in human-specific *Bacteroides* concentrations was observed in the CDS unit. This could indicate that the CDS unit serves as growth reactor for human-specific *Bacteroides*, or that a source of human fecal pollution is present between the 2 sampling points. The concentration profiles of *E. coli* and *Enterococcus* did not follow the one for human-specific *Bacteroides*, again indicating that these FIB are not related with human fecal pollution in the drain system. For *E. coli* there seemed to be a source between location

CO and HC but again, none of the other indicators followed the same trend. The *Enterococcus* concentrations increased steadily in the drain. Overall, the sources for human-specific *Bacteroides*, *E. coli* and *Enterococcus* were different in the drain system under study.

3.3.4 Dispersion coefficient

3.3.4.1 Taylor's analysis

A Gaussian model was used to calculate the dispersion coefficient:

$$S(x,t) = \frac{M}{A\sqrt{4 \cdot \pi \cdot K_x \cdot t}} \exp\left(- \frac{(x - V_x \cdot t)^2}{4 \cdot K_X \cdot t} \right)$$

With: S(x,t) = Concentration of tracer at distance x at time t

M = mass of tracer injected (g)

A = cross-sectional area of channel (m^2)

V_x = cross-sectional averaged velocity ($m\ s^{-1}$)

K_x = longitudinal dispersion coefficient ($m^2\ s^{-1}$)

t = time (s)

The average measured and predicted fluorescence were plotted versus time. M, V_x and K_x were adjusted until an approximate best fit between Gaussian and experimental data was obtained (Fig. 3-5). The fit is good except for some tailing of the experimental data, due to the occurrence of dead zones. The final predicted values are K_x = 0.025 m^2/s, and V_x = 0.014 m/s.

3.3.4.2 Chatwin's transformation

Taylor's analysis (based on Fickian model) predicts that only concentration versus distance profiles are Gaussian. The Chatwin's transformation can be used when measuring concentration versus time data at a fixed site. Data analysis includes plotting

$$S^* = \sqrt{t \cdot \ln\left(\frac{S_{max}\sqrt{t_{max}}}{S\sqrt{t}}\right)} \text{ versus } t$$

Where S_{max} = the peak concentration which occurs at time t_{max}. Both S_{max} and t_{max} can be estimated from experimental data. For $t > t_{max}$, the transformed y values are to be taken negative.

This plot yields a straight line with slope $-\frac{V_x}{2\sqrt{K_x}}$, and intercept $\frac{x}{2\sqrt{K_x}}$ (Fig. 3-6)

It is recommended (5) to only use the linear part of the plot in estimating K_x, yielding a value of 0.0144 m^2/s. As some measure of control sensitivity analysis, the total dataset was included, yielding a higher estimate of 0.0584 m^2/s. The estimated average velocities V_x were 0.0143 – 0.0148 m/s.

3.3.4.3 Rapid Estimation Method

Rutherford (5) presents a rapid estimation method for K_x, which does not require *a priori* knowledge of the tracer mass, the complete tracer profile and is robust to tracer loss. The method is based on determining the S_{max}, t_{max} and the time (t_s) during which a concentration S_c is exceeded. The equation

$$K_x \approx \frac{\left(\frac{t_s \cdot x}{4 \cdot t_{max}}\right)^2}{t_{max} \cdot \ln\left(\frac{S_{max}}{S_c}\right)}$$

is evaluated for different values of S_{max}/S_c (e.g. 1.4, 1.9, 2.8, 3.9 in this case).
The average value obtained for K_x = 0.0162 m^2/s.

3.3.5 Sediment study

FIB results from sampling sediments and overlying water suggested that enterococcus and E. coli concentrations were at similar orders of magnitude, perhaps from the influence of pore waters on what otherwise might be considered solids-

associated signals (Table 3-1). There seemed to be no conclusive pattern in this data, and a replicated study would need to be undertaken to more definitively characterize storage.

Human-specific *Bacteroides* markers were only found in the Gutierrez water and sediments samples, and the downstream of the drain water sample. However, the targets were only detected in one of six or nine analytical replicates, indicating that the sample concentration is very close to our limit of quantification for this method (Table 3-2).

3.4 Conclusions

The drain is a significant source of human-specific *Bacteroides* into Mission Creek during dry weather. It is possible that the *Bacteroides* grow in the CDS unit, so their input in the Creek is not necessarily related to human fecal pollution. However, more work is needed to verify this. After release in the creek, FIB concentrations appear to decrease with different rates among the FIB. More intensive sampling and different approaches (e.g. using dialysis bags for determining decay rates) are needed to determine decay rates of FIB in the field.

The 3 methods used for estimating K_x yield comparable results, between 0.014 – 0.025 m^2/s. Using Chatwin's transformation a higher limit of 0.058 m^2/s was obtained. The estimates are in agreement with published values on rivers with similar flow rates. The minimum and maximum estimates should be compared in the diffusion-advection model to implement the uncertainty of the K_x estimate.

Based on the results from the current study, a few recommendations can be made for similar studies in the future:

1. It is absolutely necessary to have reliable flow rate data. More efforts should be made to measure these in case we don't want to rely on stage data.
2. Incomplete mixing in the creek downstream of the drain outfall may be important and probably causes non-uniform cross-sectional FIB concentrations. The extent of mixing could be quantified by adding Rhodamine WT in the drain water.
3. Temporal variations are probably important, also on a short time scale (e.g. less than hours). Sampling strategy should be adjusted to avoid effects of these variations. This may include timing upstream and downstream sampling

according to the water velocity. Alternatively, composite samples (e.g. over 1 hour) can be taken using autosamplers.

4. Assuming complete cross-sectional mixing allows us to use analytical solutions for the 1-D diffusion advection equation, including first-order degradation. This simplifies calculations greatly. Possibly there are solutions for the 2-D model, too.
5. Perhaps a 2-D model is needed because: concentrations at/just downstream of drain cannot be assumed to be the same for the total cross-sectional area. If the assumption is made, the total load is overestimated. This will have important consequences when comparing with downstream locations where complete mixing occurred. Alternatively, a 1-D solution is used, but 3 concentrations are measured over cross-section (see dye study).

3.5 References

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Table 3-1: FIB results for Phase 3 Haley sediment study. TC = total coliform, *E. coli* = *Escherichia coli*, Ent = enterococci (IDEXX).

Description	TC (MPN/100 mL)	*E. coli* (MPN/100 mL)	Ent (MPN/100 mL)
Gutierrez water	>24196	960	1553
downstream Haley water	>24196	801	960
near Haley drain water	24196	4611	1515
upstream Haley water	>24196	1421	689
Cota water	>24196	2613	717
Gutierrez sediment	>48392	4978	<20
downstream Haley sediment	>48392	1640	150
near Haley drain sediment	>48392	1518	20
upstream Haley sediment	>48392	1970	476
Cota sediment	>48392	1476	746

Table 3-2: Human-specific *Bacteroides* qPCR results for Phase 3 Haley sediment study. Average and SE values for water samples are targets/L, and targets/g wet for sediments. Number of replicates refers to analytical replicates (each sample was run in triplicate on every plate).

Description	avg.targets/L (or g wet)	SE targets/L (or g wet)	# of replicates w/target	total # of replicates
Gutierrez water	6.8E+03	6.8E+03	1	6
downstream Haley water	3.3E+03	3.3E+03	1	9
near Haley drain water	0.0E+00	0.0E+00	0	6
upstream Haley water	0.0E+00	0.0E+00	0	6
Cota water	0.0E+00	0.0E+00	0	6
Gutierrez sediment	1.7E+03	1.7E+03	1	6
downstream Haley sediment	0.0E+00	0.0E+00	0	6
near Haley drain sediment	0.0E+00	0.0E+00	0	6
upstream Haley sediment	0.0E+00	0.0E+00	0	6
Cota sediment	0.0E+00	0.0E+00	0	6

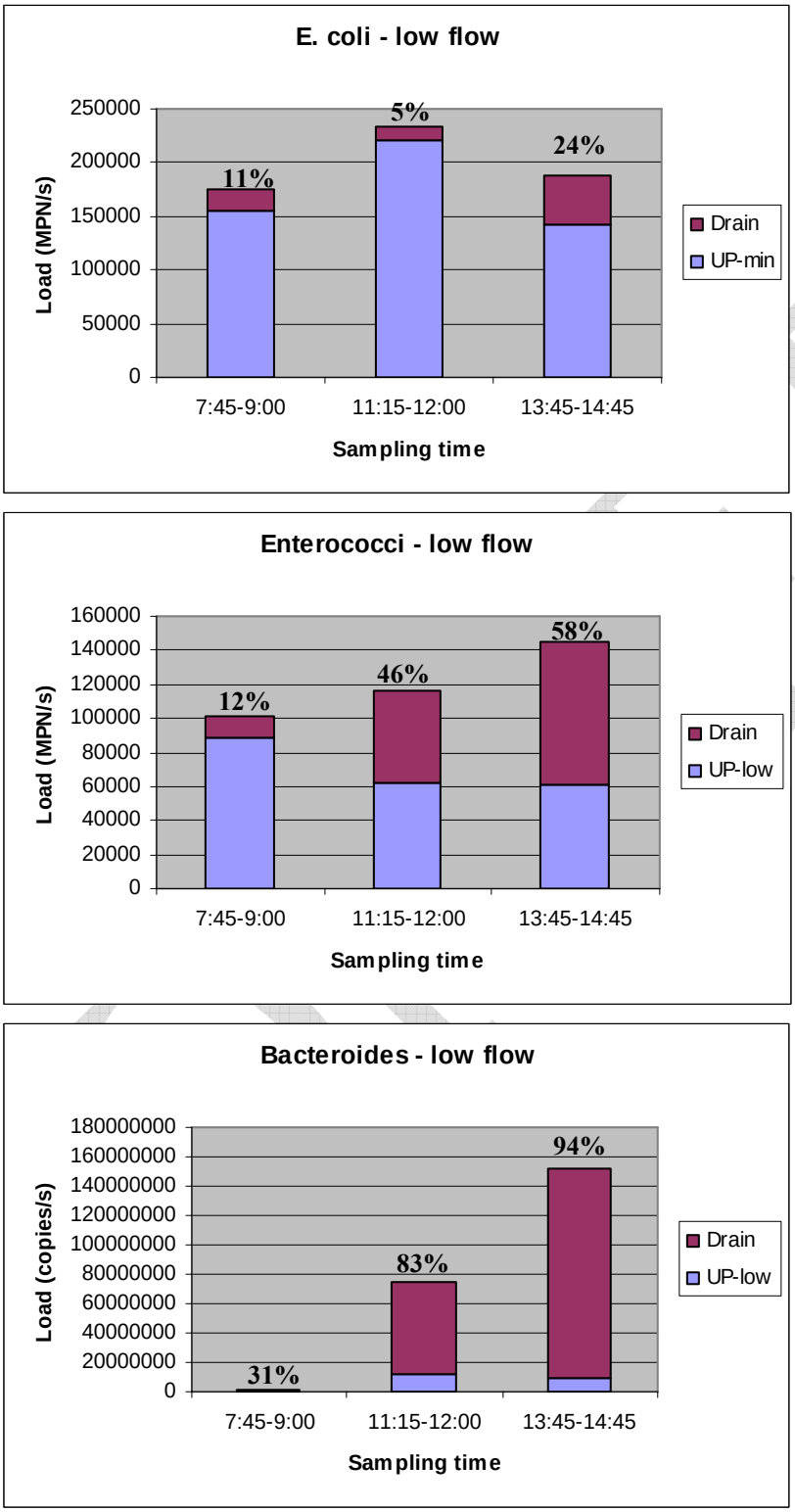
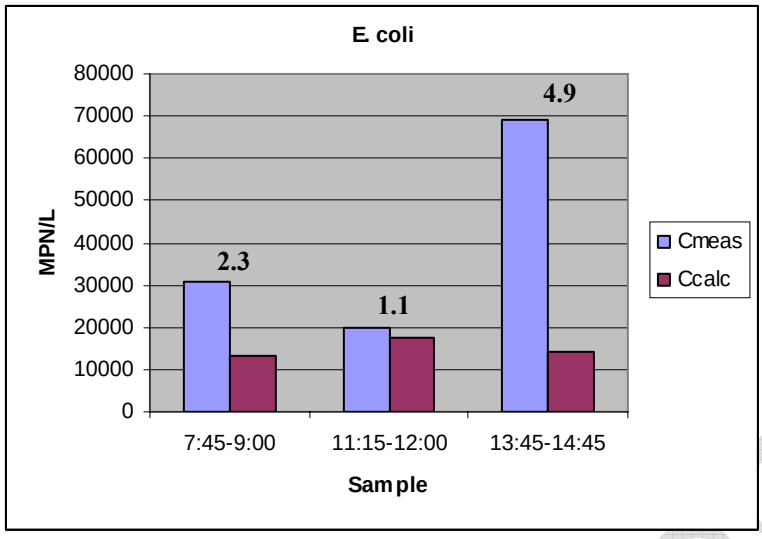
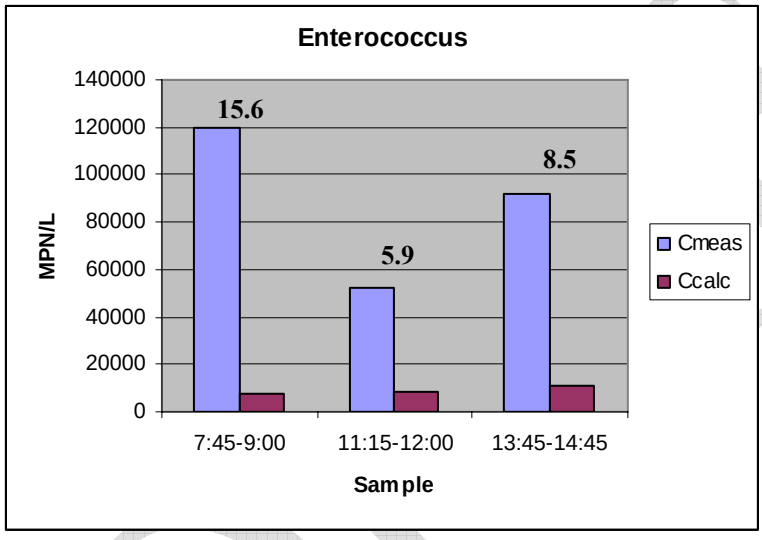


FIGURE 3-1: FIB loads in Mission Creek upstream from drain (UP) and in the drain (Drain), resulting in the total calculated load downstream of the outfall. The percentage contribution of the drain to the total FIB load downstream is indicated above the bars.





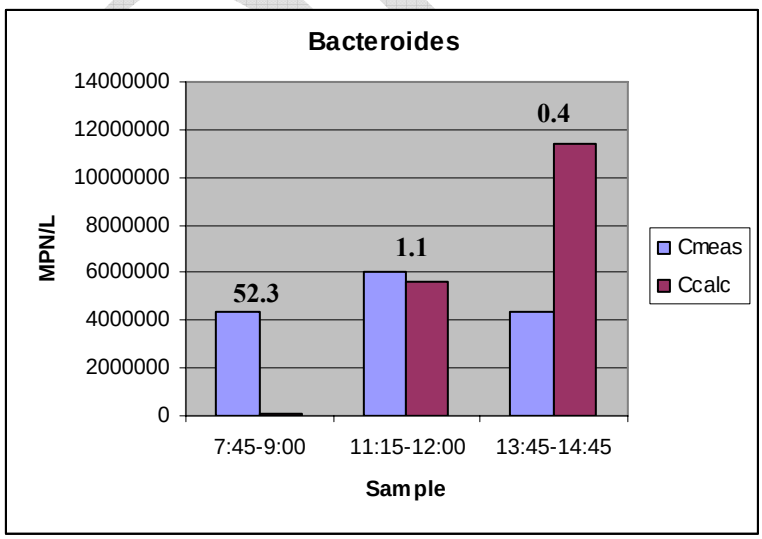
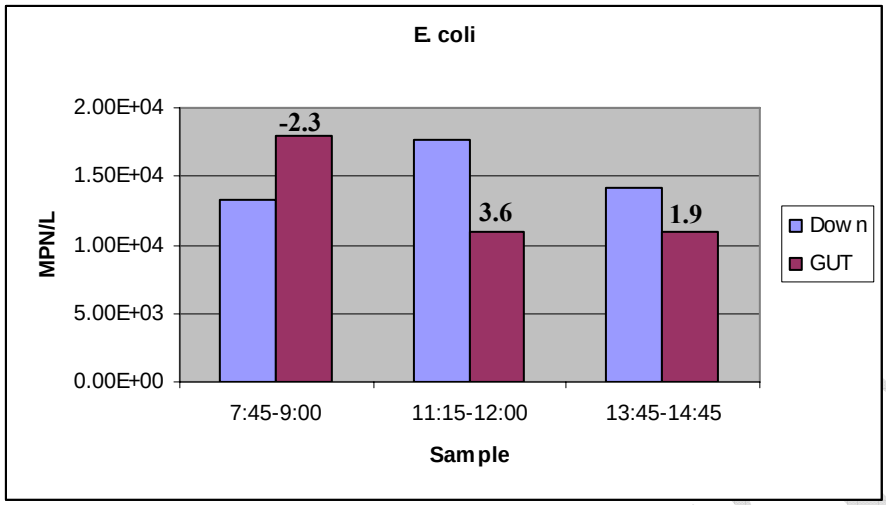
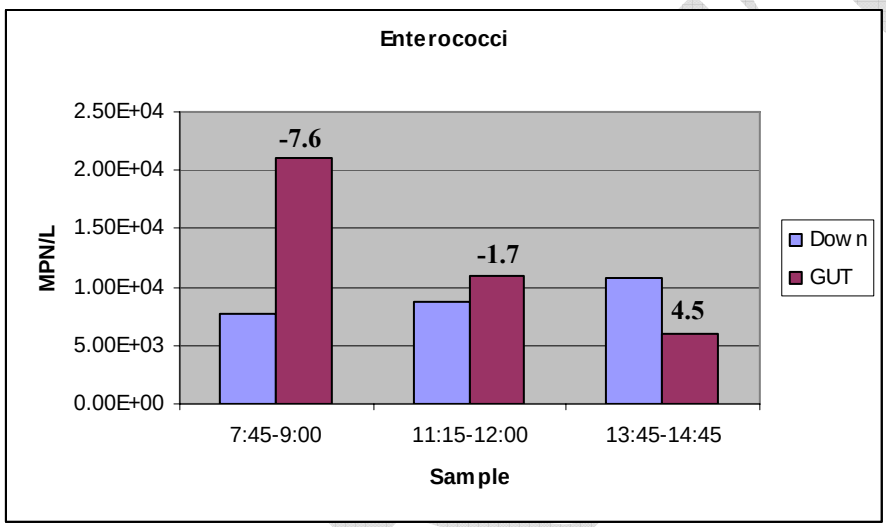


FIGURE 3-2: Calculated and measured FIB concentrations just downstream of the drain. Ratios are indicated above the bars.





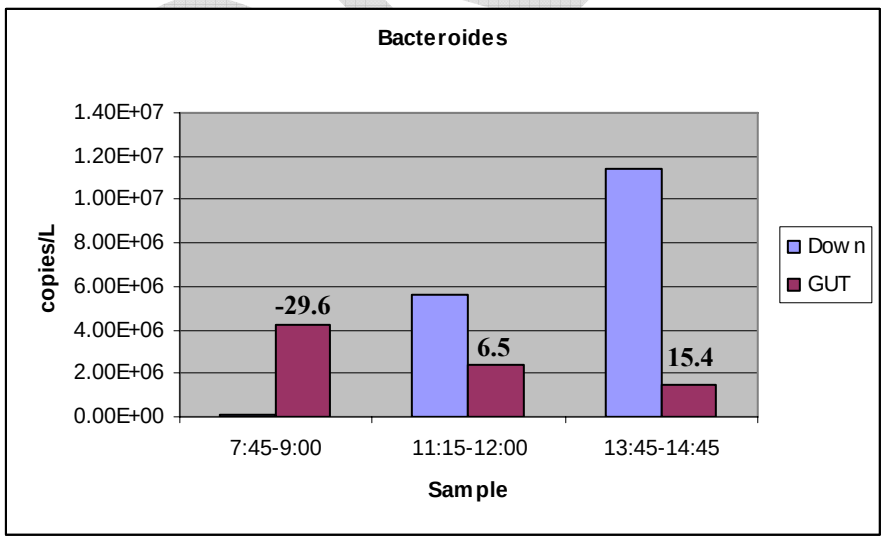


FIGURE 3-3: FIB concentrations downstream of drain outfall at distance of 20m (Down) and 180m (GUT). Calculated 1st order decay rates are included above the bars (day^{-1}).

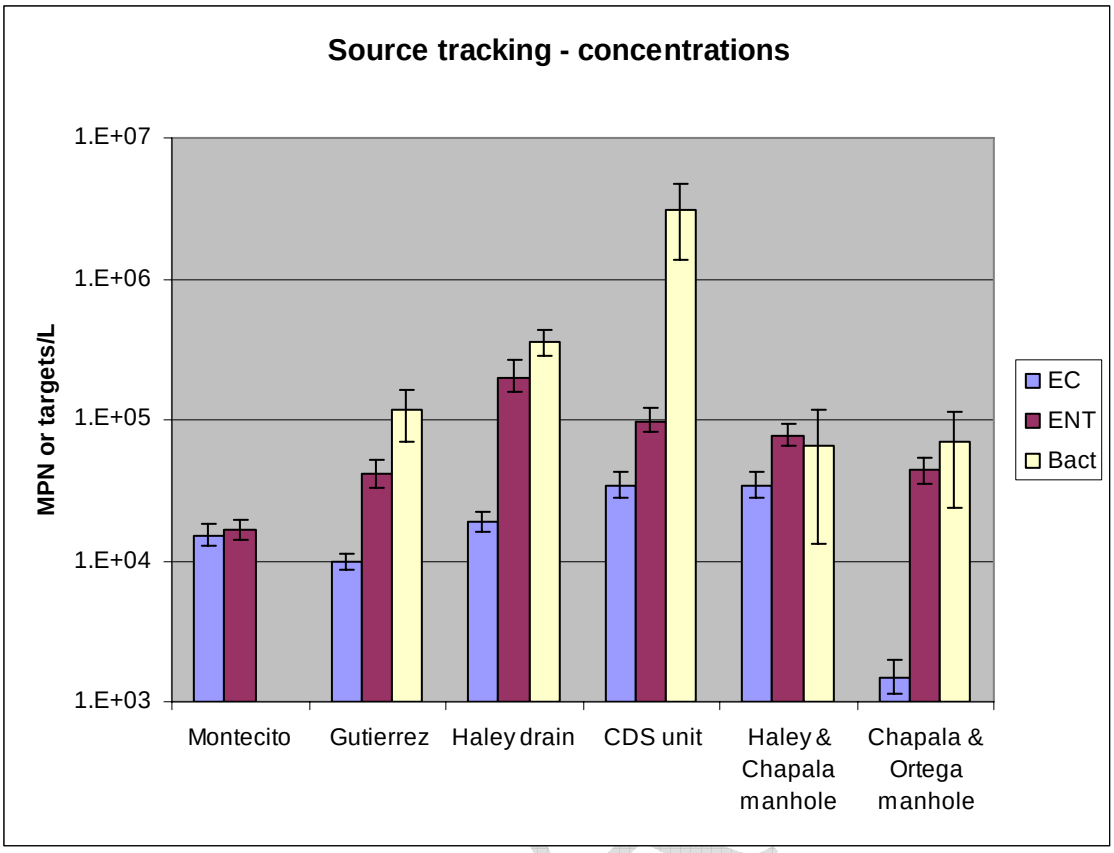


FIGURE 3-4: FIB concentrations at different locations in the drain and downstream of the drain.

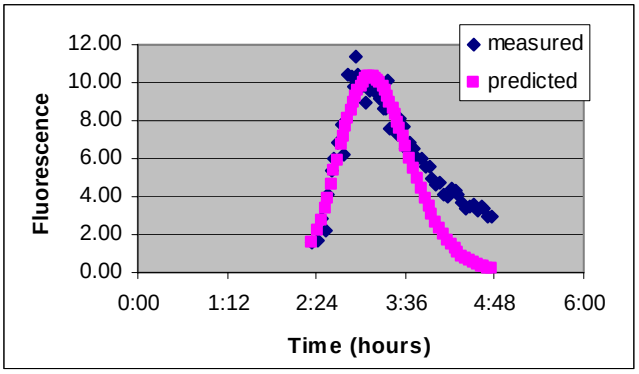


FIGURE 3-5: Best fit between measured and predicted fluorescence versus time.

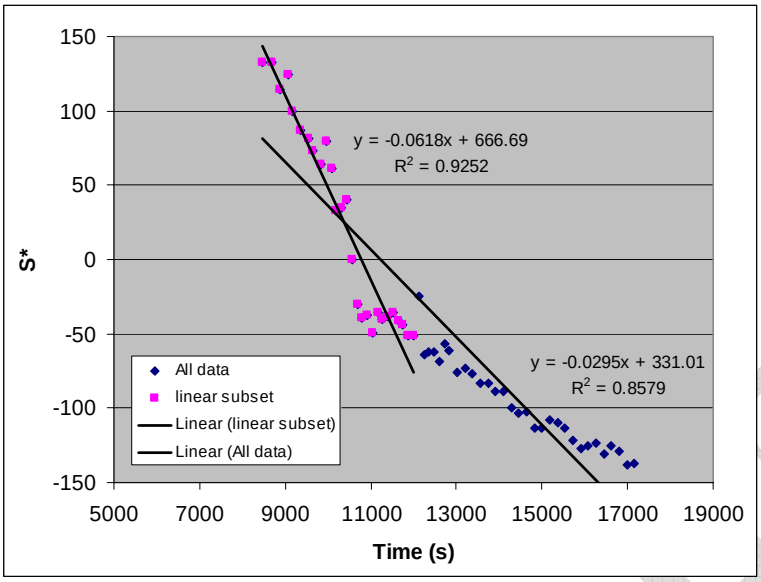


FIGURE 3-6: Estimation of longitudinal dispersion using Chatwin's transformation.

Chapter 4: Microbiological Quality of Storm Flow in Two California Coastal Creeks (Phase II Storm Study)

4.1 Introduction

In southern California shedding of fecal indicator bacteria from urbanized watersheds routinely triggers swimming advisories at coastal saltwater beaches. The high percentage of impervious surfaces in urbanized areas reduce the infiltration of rainfall, and therefore such areas are especially sensitive to the impact of storm water runoff on microbial beach water quality. Not only have several studies indicated that the loads of fecal indicator bacteria in storm water runoff are orders of magnitude higher during rainfall than during dry weather, rainfall and runoff have also been implicated in water-borne disease outbreaks within the USA (Shehane, Harwood et al. 2005).

The overall goal off this study was to assess the impact of rainfall on the concentrations and loads of fecal indicator bacteria in two highly urbanized watersheds in Santa Barbara, CA, and to determine if human waste could enter the storm water collection system. In order not to overwhelm wastewater treatment plants during rainfall, storm and sanitary sewers are generally separated in southern California. As the American Society of Civil Engineers grades the sanitary sewer infrastructure, including collection systems, in the US as poor, there is a risk that the separation of the collection systems does not guarantee that sewage does not enter open channels and creeks. We already found evidence that traces of human fecal waste enter the storm drains and urban creeks in Santa Barbara during dry weather (see Chapter 2). Now we aim to investigate if the situation is similar during wet weather.

4.2 Materials and Methods

4.2.1 Site and sampling design

Water samples were taken in two watersheds: Arroyo Burro (AB) and Mission Creek (MC). A schematic of the sampling locations is provided in Fig. 4-1, sample IDs

are summarized in Table 4-1. A map of the watersheds and approximate sampling locations is provided in Chapter 2, Figure 2-1.

Water samples were taken during a series of rainstorms in the Santa Barbara area, between 12/31/05 and 1/2/06. In Fig. 4-2, an overview of precipitation in the study area, and discharge measurements for MC at MON are shown, together with the timing of the samples. Based on the rain intensities, 4 rain events were identified, indicated as E1 and E2a-c. In Fig. 4-3, the discharge of MC at MON, and AB at CLIFF are compared. Precipitation was measured at the County Flood Control building on Anapamu Street (sensor 234), at 15 minute intervals. Discharge measurements at MON, RN and CLIFF were provided by the Santa Barbara Coastal Long Term Ecological Research project (SBC LTER).

Two sets of samples were collected in this study. First, for the temporal study, samples were taken at 4 locations in MC (HALDR, ANA, MON, LCH), at several times during the series of rainstorms. The timing of these samples is indicated in Fig. 4-2 (S0 to S4). For HALDR, ANA and LCH, S2 - S4 were pooled samples, consisting of 4 x 500 ml samples, taken on regular intervals and pooled. The length of the bar in Fig. 4-2 indicates the time frame during which the composite samples were taken. At MON, however, more samples were taken. Instead of one pooled sample S2, 4 samples were taken during the same time period. S2a-c were 2L grab samples, while S2d was a pooled sample consisting of 2 x 500 ml sample, taken 1 hour apart . Samples S3 and S4 at MON were similar as for the other locations. Second, for the spatial study, samples of both watersheds were collected on 12/31/05, between 14h30 and 15h20 in MC (= S1), and between 15h45 and 16h40 in AB. All samples were taken from down- to upstream. All sampling sites of each watershed were sampled, except for ANA.

4.2.2 Sampling and sample analysis

For water sampling, 2 L grab samples were taken, except for the pooled samples where smaller volumes were combined. Bulk water total coliforms, *E. coli* and enterococci concentrations were determined using the Colilert and Enterolert Quanti-Tray/2000 assays, by adding the appropriate dilution of the water samples to the test

media. Planktonic bacterial concentrations were determined by filtering the water samples using 3 μm Nucleopore membrane filters (Whatman, Maidstone, UK), and analyzing the filtrate using the same FIB assays.

DNA extraction was performed for the planktonic and particle-associated fractions for some samples. The particle-associated fraction was obtained by collecting the 3 μm filters, while the planktonic fraction was collected on 0.22 μm filters (MoBio, Carlsbad, CA, USA) after the first filtering step. If total bacterial DNA was extracted, cells were immediately collected on 0.22 μm filters prior to extraction. All filters were extracted using the UltraClean Water DNA Kit (MoBio, Carlsbad, CA, USA), as described before. Further DNA processing, PCR and TRFLP were performed as described before. The Primer 6 software (PRIMER-E Ltd) was used to perform non-metric multidimensional scaling (MDS), based on normalized peak heights in the TRFLP profiles.

4.3 Results

4.3.1 Flow versus Rain Intensity Patterns

Fig. 4-2 shows the rain intensity near the MON sampling station during the rain events occurring during this study period, together with the discharge measurements at two locations in Mission Creek. Two major rain events occurred during the sampling period. Event E1 consisted of a short but intense rainy period. The period with a rain intensity exceeding 2 mm h^{-1} lasted for 3.5 hours. During E1, the discharge at MON followed the rain intensity closely, with a short lag period and some tailing, but at RN almost no flow increase was observed. The second rain event consisted of 3 separate events: E2a, E2b and E2c. These events (> 2 mm h^{-1}) lasted 4.5, 5.75 and 2.25 hours, respectively, with lower intensities than observed during E1. The discharge at MON still responded to the rain intensity during E2a, again with a 1 - 2 hour lag period and some tailing. Also, no flow increase at RN was observed during E2a. While the precipitation intensity and duration for E2b and E2c were similar as for E2a, the discharge response at MON and RN changed. The discharge curves at MON didn't reflect the precipitation

patterns anymore, and more tailing was observed. Also, the discharge at RN increased significantly, until it almost equaled the MON discharge at the end of E2c.
A comparison of the discharge data for AB and MC are shown in Fig. 4-3. Discharge at CLIFF was between 0.72 – 1.33 $m^3 s^{-1}$ when the AB samples were taken, during the late phase of E1. The discharge patterns were similar to the ones in MC at MON, but the peak flows were 2 – 3 times lower.

4.3.2 FIB Concentrations: Spatial Study

In order to obtain an overview of the spatial variation of fecal indicator bacteria (FIB) concentrations and to possibly identify FIB hot spots during wet weather, several sites of Mission Creek and Arroyo Burro watersheds were sampled during the late phase of E1 on 12/31/05 (= sampling time S1). The FIB concentrations are summarized in Table 4-2, and dry weather data were added for comparison, where available. Fig. 4-1 shows a schematic of the sampling locations in the two watersheds, and FIB concentrations for all locations separate.

In MC, two locations had high FIB concentrations: RN and BP. The remaining samples (grouped as "lower creek") contained lower FIB concentrations, but all in a similar concentration range. The HALDR and MOC locations were not included with the lower creek samples. HALDR contained similar FIB concentrations as the lower creek, because a different relation to the dry weather FIB concentrations was observed. The lower creek samples contained higher EC and ENT concentrations during wet weather. The Haley drain EC concentrations were also higher during wet weather, but this was not the case for ENT. The latter concentrations were similar during dry and wet weather. The MOC FIB concentrations during wet weather were also to the lower creek concentrations, but much higher than during dry weather.

In AB, the FIB concentrations were similar for all locations, except for the ocean sample, where much lower FIB concentrations were observed. Again, compared to dry weather concentrations, higher concentrations were present.
In none of the samples of this study, human-specific *Bacteroides* markers were found.

4.3.3 FIB Concentrations: Temporal Study

In order to obtain the change in FIB concentrations during two storm events, water samples were taken just before the rainstorm on 12/31/05, and at several times during the following wet period for selected locations in MC. The FIB concentrations, precipitation and flow (MON only) are summarized in Fig. 4-4.

For MON and LCH, wet weather was related with elevated FIB concentrations. In MON, TC concentrations increased from 27300 MPN 100 ml^{-1} for S0, to 68670 – 155310 MPN 100 ml^{-1} for samples S1-S4. Concentrations of EC increased from 3090 to 5200 – 20460 MPN 100 ml^{-1}, and ENT concentrations from 730 MPN 100 ml^{-1} to 10170 – 19180 MPN 100 ml^{-1}. In LCH, the TC, EC and ENT concentrations increased from 30760, 630 and 100 MPN 100 ml^{-1} for S0, to 141360 - 241960, 9090 – 18600 and 8570 – 26130 MPN 100 ml^{-1} afterwards.

In HALDR and ANA, however, no consistent increases in FIB concentrations occurred during wet weather. For HALDR, the TC concentrations were between 39900 and 241960 MPN 100 ml^{-1} for all samples, but the lowest concentrations were observed for S2. The EC concentrations were between 4500 and 23300 MPN 100 ml^{-1}, except for the peak at S1 of 64400 MPN 100 ml^{-1}. All EC concentrations during event E2 were lower than before the rain. The ENT concentrations remained in the rather narrow range of 4100 – 10170 MPN 100 ml^{-1} during the entire period. For ANA, the highest FIB concentrations were consistently measured before the rain, with TC, EC and ENT concentrations of 241960, 17820 and 23820 MPN 100 ml^{-1}, respectively. During E2, FIB concentrations were in the range 64800 – 129970 MPN 100 ml^{-1} for TC, 4100 – 6370 MPN 100 ml^{-1} for EC, and 9050 – 17100 MPN 100 ml^{-1} for ENT. No concentrations were available for S1.

In none of the samples of this study were human-specific *Bacteroides* markers found.

4.3.4 Particle-Associated Bacteria in Mission Creek

All S0 and S1 water samples in MC were analyzed for total FIB and planktonic FIB concentrations (Fig. 4-5). Total and planktonic FIB concentrations were always

similar, indicating that only a small fraction of the total FIB is particle-associated. The percentages (mean ± standard deviation) of planktonic FIB concentrations were 100 ± 29%, 81 ± 16% and 84 ± 42 % for TC, EC and ENT, respectively.

The correlation coefficients between the total and planktonic FIB concentrations are high (0.99 for TC and EC, 0.97 for ENT) and strongly significant ($p < 0.01$). Scatter plots are shown in Fig. 4-6. Paired t-tests indicated no significant differences ($p > 0.05$) between bulk and filtered samples for TC and ENT. However, for EC a significant difference was observed ($p = 0.04$).

4.3.5 Correlations among FIB

Correlations between pairs of FIB (TC, EC, ENT) were investigated for the whole dataset (Fig. 4-4 – 4-7, Table 4-3). The FIB during wet weather were all strongly and positively correlated, with high significance ($p < 0.01$). Scatterplots reveal that correlations were real, and not biased, e.g. by outliers.

Correlations between FIB were also calculated for each site during wet weather, in order to determine if the correlations are dependent on the location. Correlation coefficients and significance of correlation are summarized in Table 4-4. All scatterplots are provided in Fig. 4-8. When all data are considered, scatterplots indicate good correlations between EC – TC, ENT – TC and ENT – EC for MON, LCH and ANA. For ANA, a good correlation can be observed between TC – EC only, while for HALDR no correlations are visible. These data are more or less supported by the statistical data in Table 4-4, although some correlations observed visually lack significance, probably because of the low number of samples. The latter is also the reason why the criteria for significance were relaxed to $p < 0.1$. The correlations observed for MON, LCH and ANA changed when the pre-storm sample (S0) was not included. First, none of the correlations were significant any more, although in MON and LCH, most FIB still showed some positive correlation. For ANA, however, correlation with ENT became negative. All FIB in the HALDR samples still showed no correlations.

4.3.6 Correlations between FIB and Rainfall

Scatterplots of correlations between all FIB and flow or precipitation intensity are shown in Fig. 4-9. At MON, ANA and HALDR no relations were found between FIB and rain or discharge. Only at LCH a positive correlation could be observed between FIB and rain, although this relation was much less when the S0 sample was excluded.

4.3.7 Microbial Community Composition of Water Samples

Non-metric Multidimensional Scaling (MDS) was used to analyze the similarity of the different samples, based on TRFLP profiling. Fig. 4-10 shows the similarity of all samples taken. This general overview indicates that ABOC and LLAG1 have quite different TRFLP profiles than the majority of the samples, which are all grouped together.

A closer look at the majority of the samples, without the outliers ABOC and LLAG1, is presented in Fig. 4-11. Several observations can be made: (1) all HALDR samples (circled) group separate, (2) several samples at time 0 (= just before storm) also group separate, (3) particle-associated and planktonic fractions form two distinct groups (shown using different symbols in Fig. 4-11), with only few exceptions. The ANOSIM test in PRIMER was used to test for differences between groups that were identified prior to seeing the data. This test was used with the data from Fig. 4-11, to determine differences between particle-associated versus planktonic TRFLP profiles, and HALDR samples versus all other samples. For both tests, ANOSIM indicated real differences, with only 1 and 3 of the 999 permutations greater than or equal to the Global R sample statistic, for particle-associated versus planktonic and HALDR versus all other samples, respectively.

Because of the distinct grouping of particle- and planktonic-associated samples, and the fact that the particle-associated fraction is quantitatively of low importance regarding FIB concentrations, we focused the TRFLP analysis further on the planktonic fractions of the water samples, excluding the previously identified outliers ABOC and LLAG1 (Fig. 4-12). This detailed MDS plot still indicated the separation of HALDR samples (group 1), but the other samples did not show any separation according to

watershed/creek. Rather than grouping according to location, the remaining AB and MC watershed samples grouped according to S0 (pre-storm, group 2) versus S1 - S4 (storm, group 3) samples. An MDS plot of the remaining S1 – S4 samples is shown in Fig. 4-13. No clear grouping according to location can be observed. Moreover, as the 2D stress is rather high (0.19), too much reliance should not be placed on the detail of the plot. It is interesting no point out, however, that the 4 MON samples (2a – 2d) group closely together, indicating that the DNA extraction/PCR-TRFLP methodology and the short-time (3.7 hours) sample variability are of minor importance compared to the variation occurring between locations and on longer time scales.

Fig. 4-12 suggested that the pre-storm planktonic microbial communities in the creeks (but not in HALDR) were different than the ones during wet weather. This was investigated more in detail, by including previous dry weather data into the analyses (Mission Creek, 06/28/05 – 06/30/05, Table 4-1). Pre-storm MC samples from 12/31/05 (MON0, LCH0, ANA0, and HALDR0) were included with the dry weather samples in Fig. 4-14. The latter figure shows that dry weather samples generally grouped separate from the wet weather samples, which included both AB and MC watershed. The spatial variation of the TRFLP profiles was also higher for dry weather than for wet weather samples, which was confirmed by analyzing the multivariate dispersion indices of each dataset from Fig. 4-14A. This dispersion index was 1.27 for MC-wet, and only 0.64 and 0.76 for MC-wet and AB-wet, respectively. Within the wet weather samples, LLAG1 and ABOC grouped separate. The samples ANA0 and LCH0 from 12/31/05 grouped with the other dry weather samples, taken between 06/28/05 – 06/30/05. However, that was not the case for MON0.

The difference between the HALDR wet and dry weather TRFLP patterns was not as large as for the other wet and dry weather samples (Fig. 4-14A), although separate grouping still occurred (Fig. 4-14B).

4.4 Discussion

4.4.1 Precipitation versus Flow in Mission Creek

The two major precipitation periods that occurred in the study area between 12/31/05 and 1/2/06 (E1 versus E2a-c) were very distinct regarding their influence on the flow in the upstream (Rocky Nook) or downstream (Montecito) reaches of Mission Creek. Events E1 and E2a were closely correlated with the discharge at MON, with a short lag period for the discharge, suggesting that mainly runoff from impervious surfaces in the highly urbanized area contributed to the MON flow. Input of older pre-event water from deeper soil horizons (Haria and Shand 2006) is unlikely because of the low infiltration due to impervious surface and storm-water infrastructure (Chang 2007). Almost no flow increase was observed at RN during E1, probably because of infiltration in the dry soil, as the upper watershed is less urbanized and the wet season had just started. So in this case, the upstream catchment area of Mission Creek Watershed was not influencing the more downstream locations. During the later rain events (E2b-c), an increasing contribution of the upstream catchment area to the MON discharge was observed. The increased flow at RN caused a decoupling of the MON flow and rainfall. Shortly after E2c, almost all flow in MON came from the upstream RN location. Overall, data indicate that the flow at MON during the earlier rain events originated from urban runoff related to the rainfall. However, during the later rain events, flow at MON was partly from rainfall in the area (during peak intensity), but mostly from upstream locations, lagging the rainfall peaks. So the area that potentially influenced the water chemistry/microbiology at MON increased during the storm. The extra input, channeled through RN, could be runoff/shallow soil flow but also groundwater that was displaced by infiltration (Haria and Shand 2006).

4.4.2 FIB Concentrations during Wet and Dry Weather in AB and MC Watersheds

For analyzing the FIB concentration data in the two watersheds, we distinguish drain samples (HALDR and HOPE), ocean water samples (MOC and ABOC) and creek water samples (remaining locations), as indicated in Table 4-2.

In general, the creek water samples of both watersheds all contained elevated FIB concentrations during wet weather. The FIB concentrations during wet weather were in the range observed before in other studies, i.e. order of magnitudes of 10^5 TC per 100 ml and 10^4 EC or ENT per 100 ml (Reeves, Grant et al. 2004; Krometis, Characklis et al. 2007). The spatial variation of FIB concentrations, from lagoon to the more upstream creek locations, was low in both creeks during wet weather, but high during dry weather. For some samples, dry weather FIB concentrations peaked near or above the wet weather concentration range, as was similarly observed by (Reeves, Grant et al. 2004). However, the low range of the dry weather FIB concentrations was always 1 – 2 orders of magnitude lower than for wet weather. These observations agree with the current knowledge that FIB concentrations in runoff increase during wet weather (Shehane, Harwood et al. 2005; Sullivan, Snyder et al. 2005; Surbeck, Jiang et al. 2006). However no simple correlation could be observed between FIB concentrations and rainfall intensity or discharge, as was also observed before (Surbeck, Jiang et al. 2006).
The ocean samples for each watershed indicated a similar trend in FIB concentrations, i.e. higher concentrations during wet weather, with even higher differences than for the creek water samples. So the elevated FIB concentrations in the creeks have an impact on the microbial water quality in the ocean's surf zone during wet weather. The MC ocean water contained higher FIB concentrations than the AB ocean water, although the creek water samples of both watersheds contained similar FIB concentrations. The MC ocean FIB concentrations were actually very similar to the ones in the lower reaches of MC, so it seems likely that the freshwater plume that entered the ocean was sampled. This is further supported by the fact that the MOC1 sample grouped together with the other MC lower creek water samples in the MDS plots. It is important to note that both beaches were in violation with the California single sample standard for ENT (> 104 MPN 100 ml^{-1}), and MOC also with the TC (> 10000 MPN 100 ml^{-1}) and EC (> 400 MPN 100 ml^{-1}, assuming EC ≈ fecal coliforms) standard. This agrees with previous work at California beaches, showing that ENT was the indicator that failed the single sample standards most often during wet weather (Noble, Moore et al. 2003), and that storm water runoff from an urban watershed can lead to very poor surf zone water quality (Ahn, Grant et al. 2005).

In contrast, the rainfall had a much smaller effect (if any) on the FIB concentrations for the drain samples. For HALDR, TC and EC concentrations were higher during wet weather, but ENT concentrations were similar during wet and dry weather, and sometimes slightly higher during dry weather. For HOPE, all FIB concentrations were similar during dry and wet weather. This suggests that the origin of the drain water is the same throughout the year, and that only the volumes increase during wet weather. This is further supported by a close grouping of all dry and wet weather HALDR samples in the MDS plots (Fig. 4-14A), especially when compared to the large differences between creek wet and dry weather samples.

The flow patterns and microbial characteristics of Mission Creek and Arroyo Burro watershed during wet weather were highly similar. First, the response of discharge to precipitation was highly similar, although higher peak flows occurred in Mission Creek (Fig. 4-3). Second, both watersheds contained similar and rather constant FIB concentrations in the lower creek reaches, and consequently the FIB loads, calculated for MON and CLIFF, were also similar. At CLIFF, the bacterial loads were 2.2×10^9 MPN s^{-1} for TC, 1.8×10^8 MPN s^{-1} for EC, and 3.5×10^8 MPN s^{-1} for ENT, while at MON, the loads were 5.7×10^9 MPN s^{-1} for TC, 5.1×10^8 MPN s^{-1} for EC, and 7.1×10^8 MPN s^{-1} for ENT. Thirdly, the microbial communities, based on TRFLP profiling, of both watersheds were highly similar (Fig. 4-12). Only the two drains that flowed into both creeks had slightly dissimilar microbial characteristics during wet weather. The EC concentrations in HOPE were lower than in the creek, but the ENT concentrations were higher. For HALDR, this was just the opposite, and relative to the creek high EC but low ENT concentrations were found during wet weather. HALDR samples also grouped separate from the MC creek samples in MDS plots, although this was not the case for HOPE. Nonetheless, the drains did not seem to influence the creek water FIB concentrations to a large extent. This ultimately indicates that land use differences between both watersheds (e.g. more high density and rural residential area in MC, and mainly open space and medium density residential area in AB, (City of Santa Barbara 2002)) did not greatly influence FIB concentrations and loadings in the lower reaches of the creeks. Therefore, the impact on the surf zone water quality during wet weather is expected to be similar for both watersheds.

The data from AB in the creek compare well with the data from another sampling event during wet weather (11/09/05) at CLIFF (see Chapter 5), when slightly higher TC (345 x 10^3 MPN 100 ml^{-1}) and EC (41 x 10^3 MPN 100 ml^{-1}), and similar ENT (21 x 10^3 MPN 100 ml^{-1}) concentrations were observed. This gave more confidence that the current wet weather FIB concentrations are representative for typical wet weather conditions. Comparing dry (10/24/05) and wet weather (11/09/05) FIB loadings at CLIFF indicated a huge increase during wet weather: from 2.0 x 10^6 to 1.2 x 10^{10} MPN s^{-1} for TC, from 3.6 x 10^4 to 1.5 x 10^9 MPN s^{-1} for EC, and 3.3 x 10^4 to 7.4 x 10^8 MPN s^{-1} for ENT. So FIB loading rates increased 6000- to 41000-fold during wet weather, indicating that the majority of the FIB loading to the ocean occurs during storm events (Reeves, Grant et al. 2004). Based on the similar behavior of both watersheds during wet weather, it is expected that the same phenomenon will occur for FIB loads from MC to the beach. Recent studies have found that beach sand can be a reservoir for FIB, and that those FIB can be transported to the overlying water (Alm, Burke et al. 2006; Beversdorf, Bornstein-Forst et al. 2007; Ishii, Hansen et al. 2007; Yamahara, Layton et al. 2007). This means that high FIB loadings during storms may contribute to impairment of beach water quality in-between storms, or even in summer, when most of the recreational activity and exposure to microbial contaminants in the ocean occurs.

4.4.3 Correlations between FIB

When concentration data for TC, EC and ENT from all wet weather samples were correlated, we found a good correlation between all FIB. This was similar to the findings of (Noble, Moore et al. 2003), who showed that FIB concentrations were strongly correlated during storms, although they found that this was not the case during dry weather. If we correlated FIB concentrations per location, correlations between the different FIB were still visible, although not always statistically significant. However, not too much importance should be attributed to the significance values in this case, as only 3 – 4 observations were available per correlation. Interestingly, correlations among the FIB were not found for HALDR. The differences in correlations among FIB for creek water

samples and HALDR samples are probably related with different sources of FIB for both sets of samples. If correlations among FIB are good for different samples, it means that all 3 FIB originate from a similar source, which would be urban runoff, and also that their fate between source and ocean is similar (e.g. similar decay rates). When no correlations are observed, as in HALDR, this points to different sources for each FIB, or very different decay rates during transport in the drain system.

4.4.4 Particle-Associated Microbial Communities

In this study we found that particle-associated FIB are quantitatively of minor importance in the Mission Creek watershed during wet weather. Only EC concentrations were significantly different between the entire water column and the planktonic fraction, with the latter containing on average 81% of EC. This agrees with previous findings from (Surbeck, Jiang et al. 2006), who found that FIB rapidly partition into the surface water as the landscape is wetted by rainfall, and are therefore not particle-associated. (Krometis, Characklis et al. 2007) found that on average 40% of the fecal indicator bacteria (TC, EC and ENT) were associated with settleable particles. The higher fraction in their study could be related with site-specific characteristics, or with the fact that the authors used a calibrated centrifugation method to separate settleable particles, whereas in this study a filtration method (3 μm cutoff) was used. Also, the authors found that the fraction of microbes associated settleable particles varied during the course of a storm, although 25th and 75th percentiles were between 25 and 60 %.

The MDS plots indicated that the particle-associated microbial communities were mostly distinct from the planktonic ones (Fig. 4-11). For the MC watershed samples at time S0 and S1, particle-associated and planktonic microbial communities were separated, whereas for the remaining samples (all AB watershed samples, MC watershed samples after S1), only total microbial communities were used. However, the similarity of most planktonic microbial community samples to the total microbial community samples indicated that the particle-associated fraction was an insignificant portion of the total microbial community.

4.4.5 Microbial Communities in Storm Water

In this study, the changes in microbial community composition between sampling locations and sampling times were used to investigate the sources of storm water in two watersheds. The logic is that sampling locations having very similar microbial communities must be influenced by the same source of water, and the microbial communities change little while being transported from one location to the other. When looking at MDS plots of all samples collected (Fig. 4-10), it is very obvious that main driver for microbial community change was marine versus freshwater, as was observed before (Bernhard, Colbert et al. 2005). However, some ocean/lagoon samples (MOC1 and MLAG1) grouped with the bulk of the creek water samples, indicating that these locations were still largely influenced by freshwater, which could be related with the high flow of Mission Creek and consequent sampling of the freshwater plume. This was also in agreement with the high FIB concentrations observed at MOC1 and MLAG, but not in ABOC. The LLAG1 sample also contained relatively high FIB concentrations, but did not group with the freshwater samples, so in that case dilution and microbial community change in salty water alone was probably not the cause of its separate grouping. The grouping among the freshwater (creek and drain) samples was investigated in separate MDS plots, which did not include the outlier ocean/lagoon samples. These more detailed MDS plots indicated that several pre-storm samples, and also all HALDR samples, grouped separate from the remaining samples.

The separation of the pre-storm samples suggested that the microbial communities during dry and wet weather in the creeks are different. This was further investigated by also including previous dry weather samples in the analysis (Fig. 4-14A). Dry weather microbial communities were very distinct from wet weather microbial communities. The change in microbial community from dry to wet weather was as large as the change from ocean/lagoon to freshwater. Not only were wet and dry weather microbial communities different, the dry weather samples were also more dissimilar among each other. Wet weather microbial communities, on the other hand were highly similar, although separated into creek and HALDR samples. The high spatial variability of the microbial communities during dry weather is in agreement with the previous findings that point sources (i.e. Haley Drain) are present in Mission Creek (see Chapter 2). The high

similarity of the wet weather samples, together with the very similar FIB concentrations at the lower reach sections, indicated spatially and temporally similar inputs to all locations in the creeks of both watersheds, and no significant point sources. The slightly separate microbial communities in HALDR and the other freshwater samples (Fig. 4-11, Fig .14B) also indicated that the HALDR input into the creek was not significant enough to change the microbial community composition in the creek, and therefore, by extension, the FIB concentration in the creek.

We argued above that the catchment area influencing the flow at MON increased during the later phases of the rain events from this study. This change was also reflected by the microbial community composition at MON. The MDS plot in Fig. 4-13 indicated a separate grouping for samples MON3 and MON4 (E2b-c), MON2a-d (E2a) and MON1 (E1) samples. This separate grouping was not observed for the LCH3-4 and ANA3-4 samples. The grouping was further confirmed in the 3D MDS plot (not shown), which had a lower stress value (0.12). However, these changes were only minor compared to the differences in microbial community composition between wet versus dry weather samples or even HALDR versus creek water samples.

4.5 Conclusions

Sampling of creek and drain waters in two watersheds during a series of storm events indicated that high FIB concentrations and FIB load were associated with wet weather, which clearly impacted the microbial water quality of the ocean water downstream of each watershed.

Detailed analysis of FIB concentrations, correlations between FIB and changes in microbial community composition indicated that:

- The FIB concentrations in the drains were similar during dry and wet weather, which was not the case in the creeks,
- The human-specific *Bacteroides* markers could not be detected in drains or in creek water samples during wet weather, although they were detected during dry weather,
- No correlations were found between FIB concentrations in HALDR during wet weather, whereas these correlations were present in the creeks,

- HALDR water had different microbial communities than the creek water, during wet and dry weather.

The combination of these observations all point to the conclusion that the water flowing in the drains had highly similar sources of traditional FIB during wet and dry weather. However, during wet weather the human-specific *Bacteroides* concentrations were diluted by storm water, not impacted by human waste. While the drains impaired microbial water quality in the creeks during dry weather, no such evidence was found during wet weather. FIB sources appeared diffuse over the watershed, and no sign of human fecal contamination was observed.

4.6 References

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TABLE 4-1: Sample IDs used for comparison of wet (12/31/05 – 1/2/06) and dry (6/28/05 – 6/30/05) weather TRFLP data. Numbers after the sample IDs for Mission Creek watershed indicate the time of sampling.

Location	ID (wet)	ID (dry)
Arroyo Burro Watershed		
Hope Drain	HOPE	
AB Creek at Modoc St.	MOD	
AB Creek at Hidden Valley	HID	
AB Creek at Cliff Dr.	CLIFF	
AB Lagoon	ABLAG	
AB surf zone	ABOC	
Mission Creek Watershed[1]		
MC Creek at Rocky Nook Park	RN1	
MC Creek at Haley St.	HALCR1	DHALCR1-3
Haley Drain	HALDR0-4	DHALDR1-3
MC Creek at Montecito St.	MON0-4	DMON1-3
MC Lagoon	MLAG1	DMLAG1-3
MC surf zone	MOC1	DMOC1-3
Laguna Channel at Chase Palm Park	LCH0-4	DLCH1-3
Laguna Creek at lagoon	LLAG1	DLLAG1-3
Old Mission Creek at Bohnett Park	BP1	WDR1-3[2]
Old Mission Creek at Anapamu St.	ANA0-4	

[1]All Mission Creek Watershed samples at times S0 and S1 were analyzed for particle-associated and planktonic fractions. Other samples were analyzed on bulk water only.

[2]samples WDR during dry weather were taken at the Westside drain, but were used for comparison with Bohnett Park at wet weather.

TABLE 4-2: Concentrations of FIB (10^3 MPN/100 mL) in the Mission Creek and Arroyo Burro watersheds, at sampling time S1 (= wet). Dry weather concentrations are added for comparison. For MC, lower creek includes samples HALCR, MON, LCH, LLAG and MLAG. For AB, creek includes samples MOD, HID, CLIFF and ABLAG.

Locations	TC		EC		ENT	
	wet	dry*	wet	dry*	wet	dry*
Mission Creek						
RN	659	n.a.	56	n.a.	272	n.a.
BP	1414	n.a.	154	n.a.	238	n.a.
HALDR	210	>24[1]	64	1.5 – 13[1]	7	2.3 – 24[1]
Lower Creek	130 - 242	5.5 - >24[2]	13 – 16	0.1 - 6.5[2]	15 - 19	0.2 - 3.9[2]
MOC	155	0.1 - 3.6[3]	18	0.02 – 0.3[3]	21	0.02 – 0.04[3]
Arroyo Burro						
HOPE	173	199 - > 242[4]	10	5.3 – 13[4]	33	9.1 – 57[4]
Creek	31 - > 242	2.4 - >242[5]	10 - 17	0.1 – 28[5]	16 - 31	0.1 – 17[5]
ABOC	3.6	0.2 – 6.9[6]	0.3	0.05 – 0.4[6]	0.2	0.01 – 0.05[6]

*n.a.: data not available

[1]data from 6/28/05 – 6/30/05, 8/2/05, 8/4/05 at HALDR

[2]data from 6/28/05 – 6/30/05, 8/2/05, 8/4/05 at HALCR, MON, LLAG, LCH, MLAG

[3]data from 6/28/05 – 6/30/05 at MOC

[4]data from 8/23/05 – 8/25/05 at HOPE

[5]data from 8/23/05 – 8/25/05 at MOD, HID, CLIFF, ABLAG

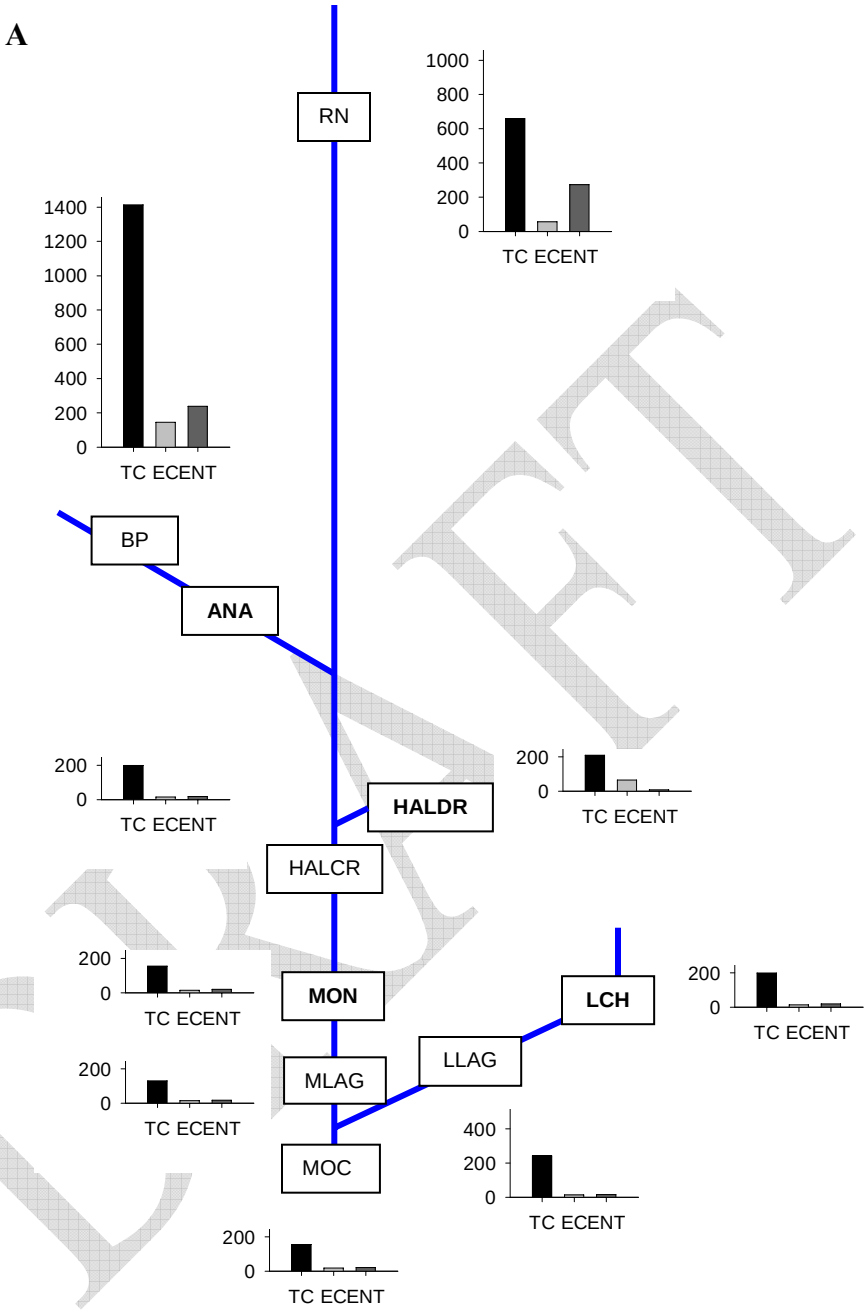
[6]data from 8/23/05 – 8/25/05 at ABOC

TABLE 4-3: Overview of Pearson correlation coefficients for non-transformed and log-transformed FIB concentrations. All correlations are significant at the p < 0.01 level.

		TC	EC
All data (Pearson)	**EC**	0.929	
	ENT	0.858	0.779
All log data (Pearson)	**EC**	0.865	
	ENT	0.794	0.832

TABLE 4-4: Pearson correlation coefficients and significance levels for non-transformed FIB concentrations, per location. Correlations were calculated for all samples (S0-S4) and excluding sample S0. Correlations significant at the p = 0.1 level are indicated with *.

		S0-S4		S1-S4	
Location	FIB pair	R^2	p	R^2	p
MON	EC-TC	0.69*	0.057	0.57	0.18
	ENT-TC	0.79*	0.02	0.58	0.17
	EC-ENT	0.42	0.31	0.05	0.92
LCH	EC-TC	0.96*	0.01	0.82	0.2
	ENT-TC	0.77	0.13	0.34	0.66
	EC-ENT	0.84*	0.076	0.56	0.44
ANA	EC-TC	0.97*	0.026	0.9	0.29
	ENT-TC	0.74	0.26	-0.37	0.76
	EC-ENT	0.79	0.21	-0.74	0.47
HALDR	EC-TC	0.51	0.38	0.51	0.49
	ENT-TC	0.45	0.45	0.72	0.28
	EC-ENT	0.06	0.92	0.093	0.91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MON
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TC ECENT
MOC
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TC ECENT

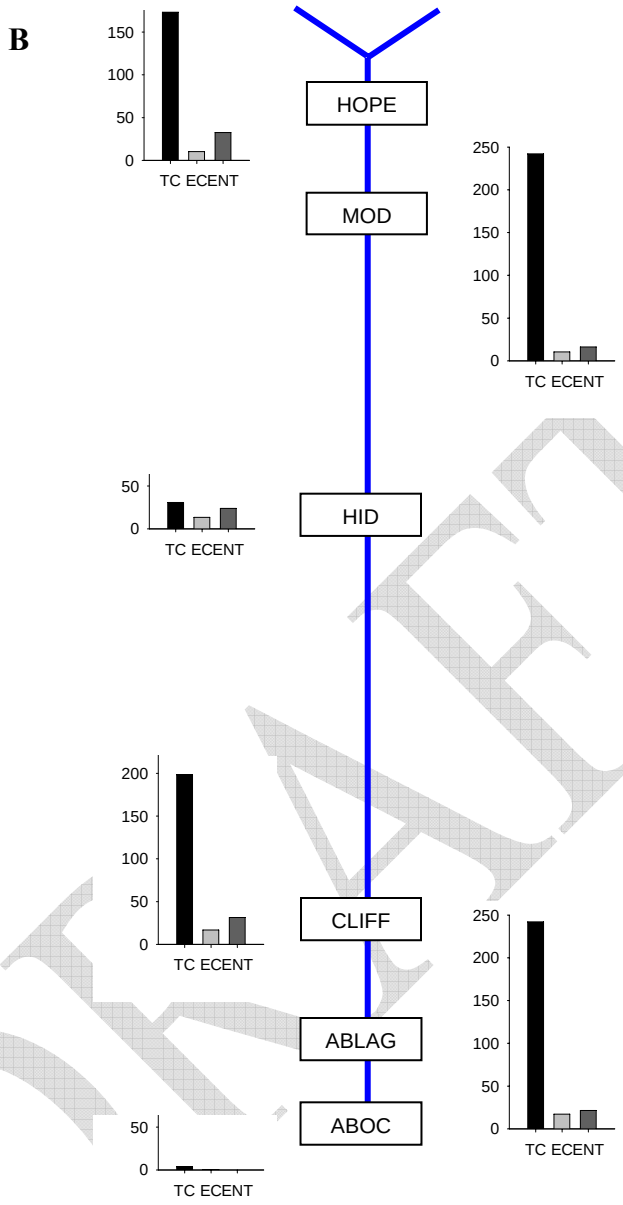

FIGURE 4-1: Schematic of the sampling locations in Mission Creek watershed (A) and Arroyo Burro Watershed (B). Locations that were sampled multiple times are indicated in bold. Total coliforms (TC), *E. coli* (EC) and enterococci (ENT) concentrations (10^3 MPN per 100 ml) are shown at each location for sampling time S1.

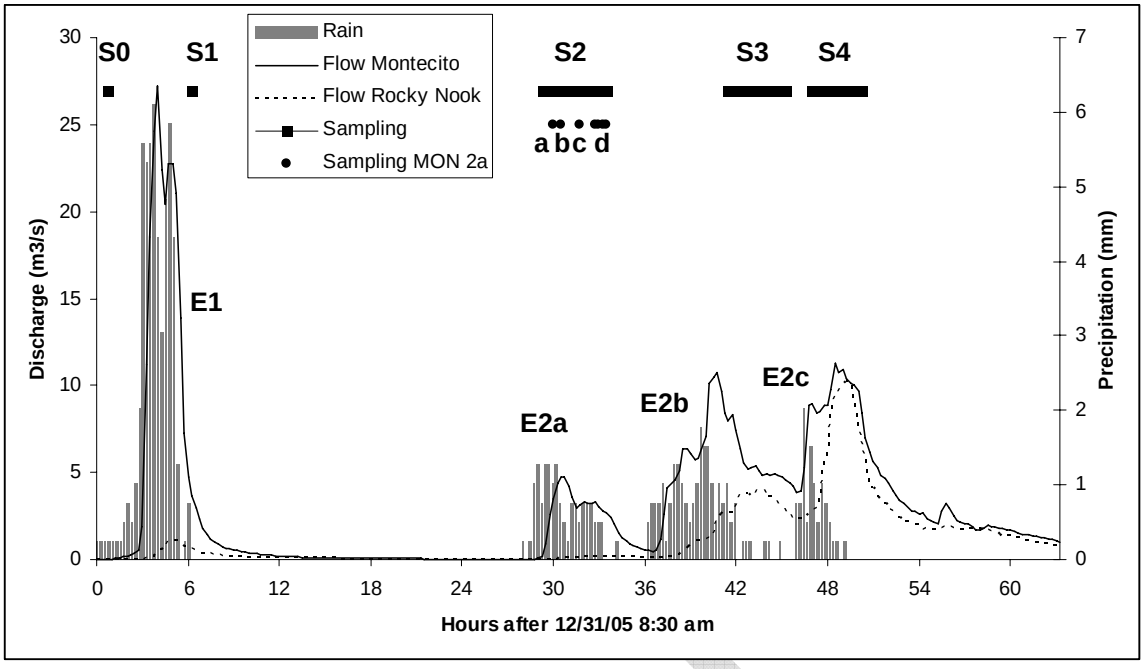

FIGURE 4-2: Comparison of rainfall intensity (15 min interval) with discharge in Mission Creek at Montecito and at Rocky Nook. Rain event are indicated with numbers E1, E2a-c. Sampling times are indicated in black squares, and numbered S0 – S4. Only at Montecito, samples were S0, S1, S2a-d, S3, S4.

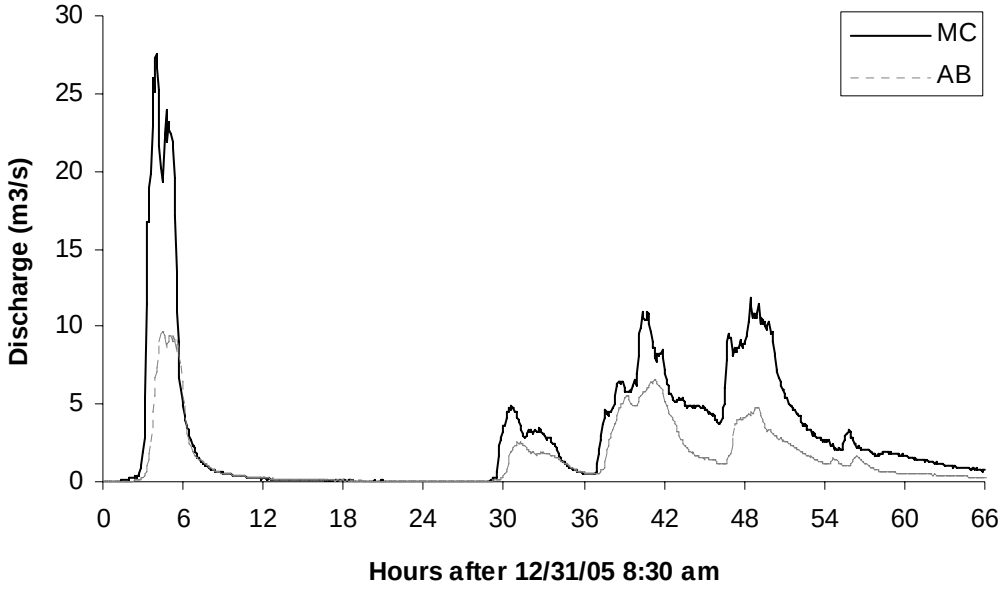

FIGURE 4-3: Comparison of discharge at MON in MC, and at CLIFF in AB.

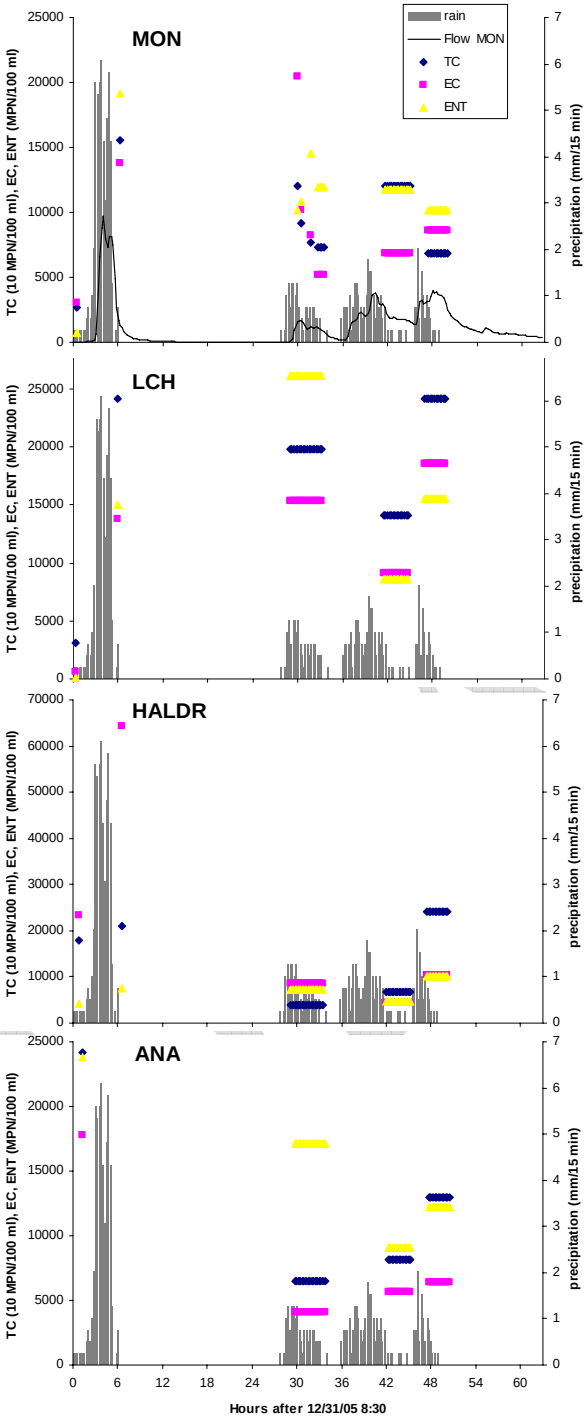

FIGURE 4-4: Temporal variation of TC, EC and ENT concentrations during two storm events, for locations MON, LCH, HALDR and ANA. Rain intensity is indicated for all samples, flow is indicated for MON only.

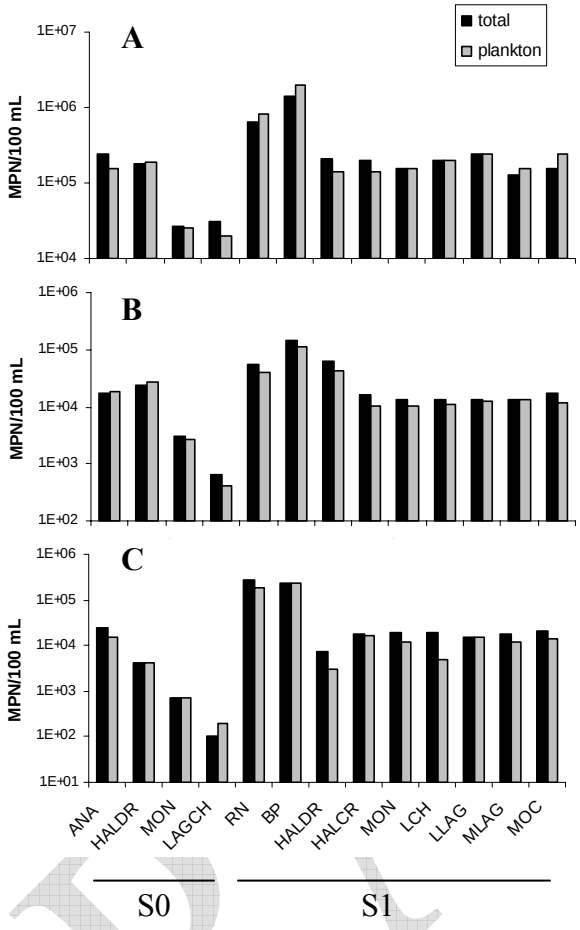

FIGURE 4-5: Total and planktonic concentrations of total coliforms (A), *E. coli* (B) and enterococci (C) in Mission Creek watershed. Samples are taken during period S0 and S1.

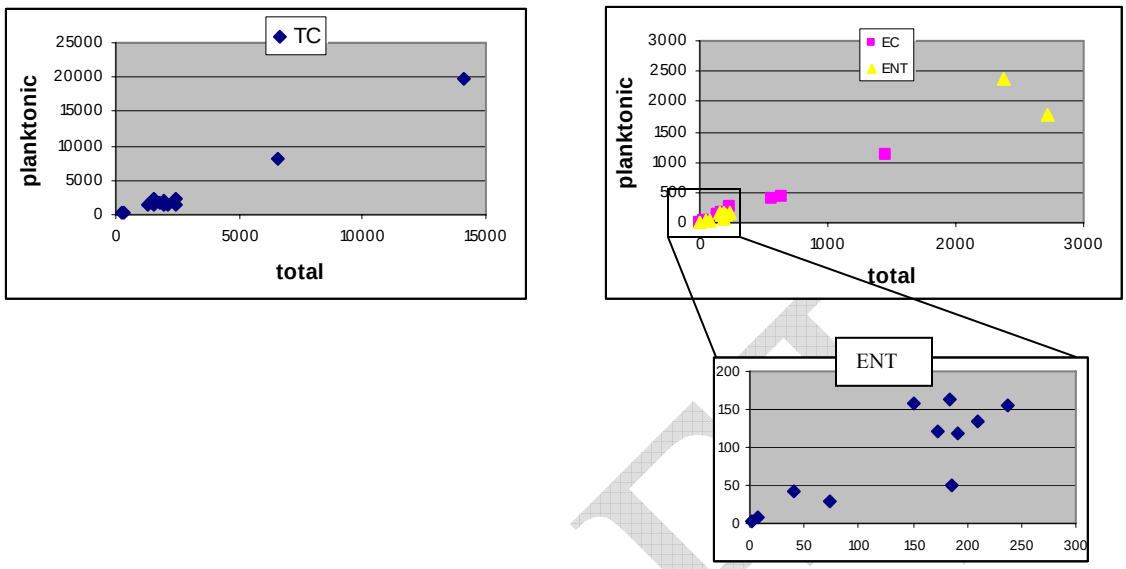

FIGURE 4-6: Scatter plots showing the relation between planktonic and total concentrations of total coliforms (TC), *E. coli* (EC) and enterococci (ENT). A detail of the ENT plot is provided in the lower concentration range.

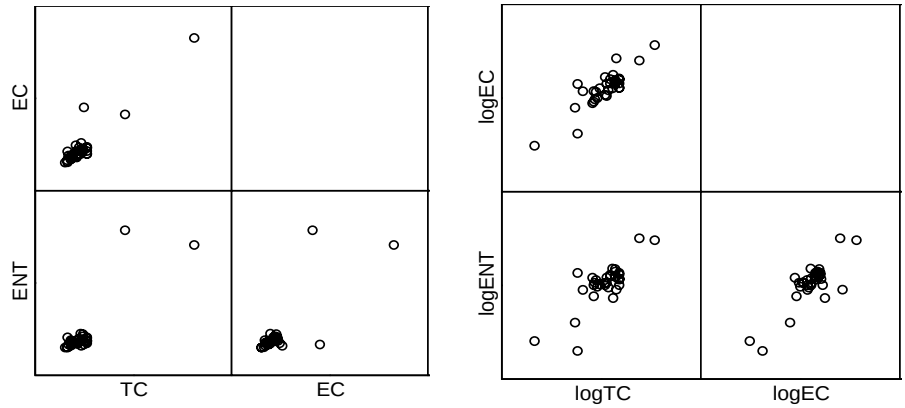

FIGURE 4-7: Scatterplots of non-transformed and log-transformed FIB concentrations for the complete wet weather dataset.

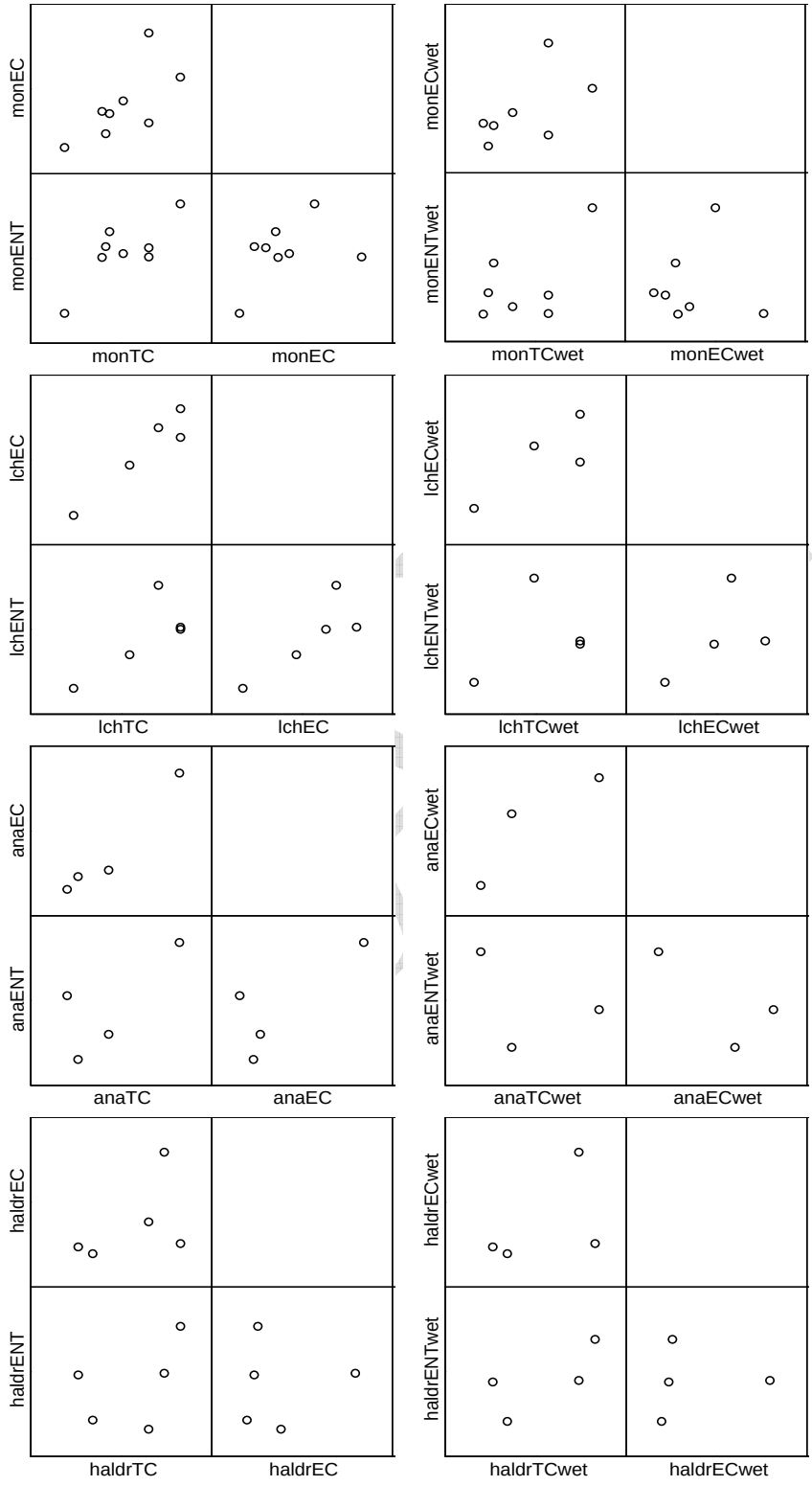

FIGURE 4-8: Scatterplots of FIB pairs for samples S0 – S4 (left graphs) and samples S1 – S4 (right graphs).

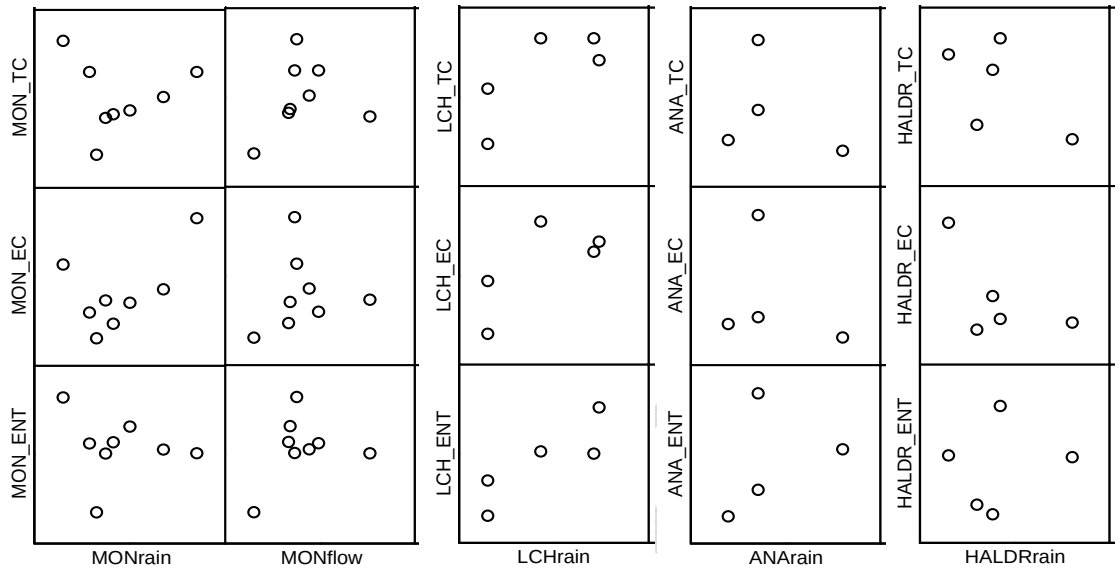

FIGURE 4-9: Scatterplots of rain intensity (all samples) and flow (MON only) versus FIB.

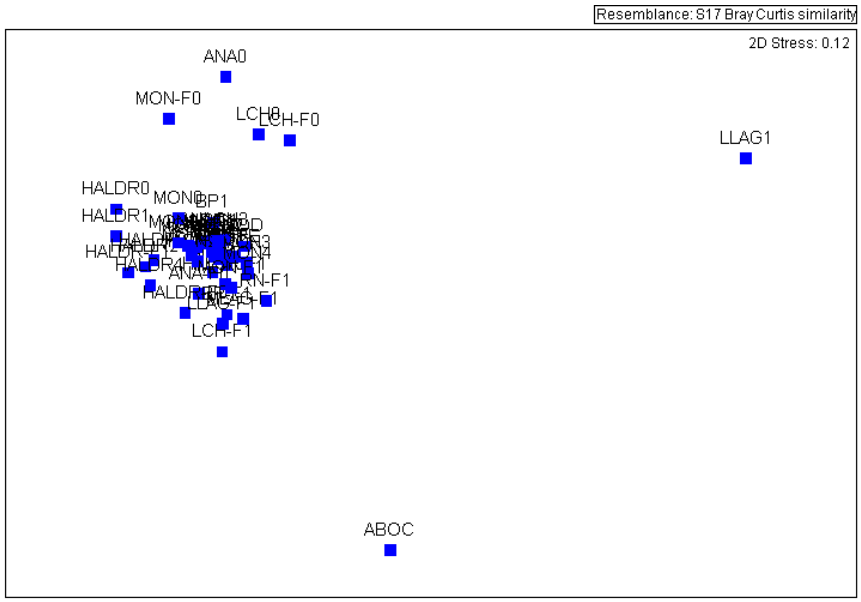

FIGURE 4-10: MDS plot of all samples, based on normalized TRFLP peak heights.

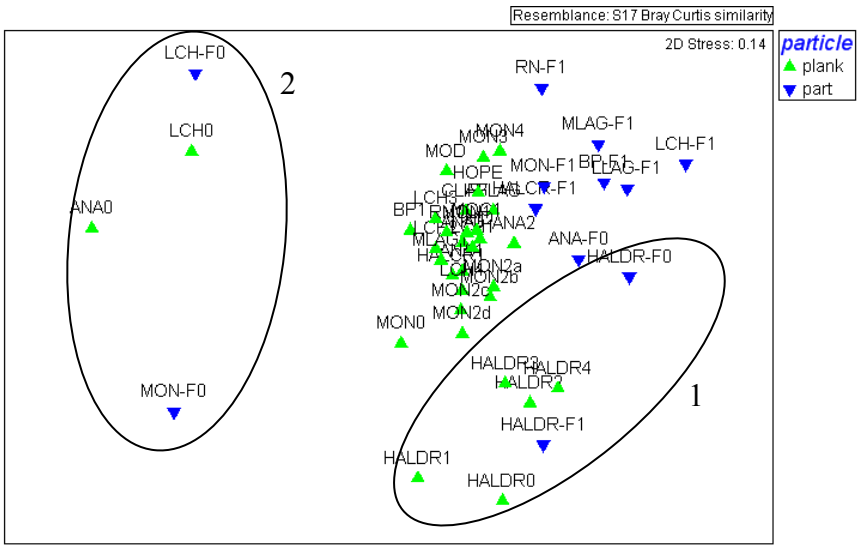

FIGURE 4-11: MDS plot of all samples, except ABOC and LLAG1, based on normalized TRFLP peak heights. Particle-associated and planktonic fractions are indicated using different symbols. Samples from HALDR (group 1) and certain S0 samples (group 2) are circled.

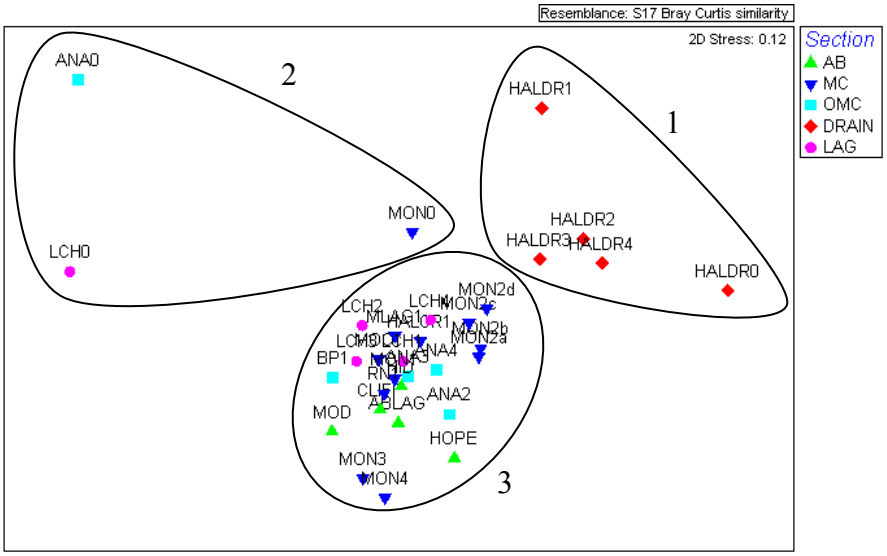

FIGURE 4-12: MDS plot of all planktonic samples, except ABOC and LLAG1, based on normalized TRFLP peak heights. The symbols grouped samples according to watershed/creek, with groups Arroyo Burro (AB), Mission Creek (MC), Old Mission Creek (OMC), Haley Drain (DRAIN) and Laguna Channel (LAG).

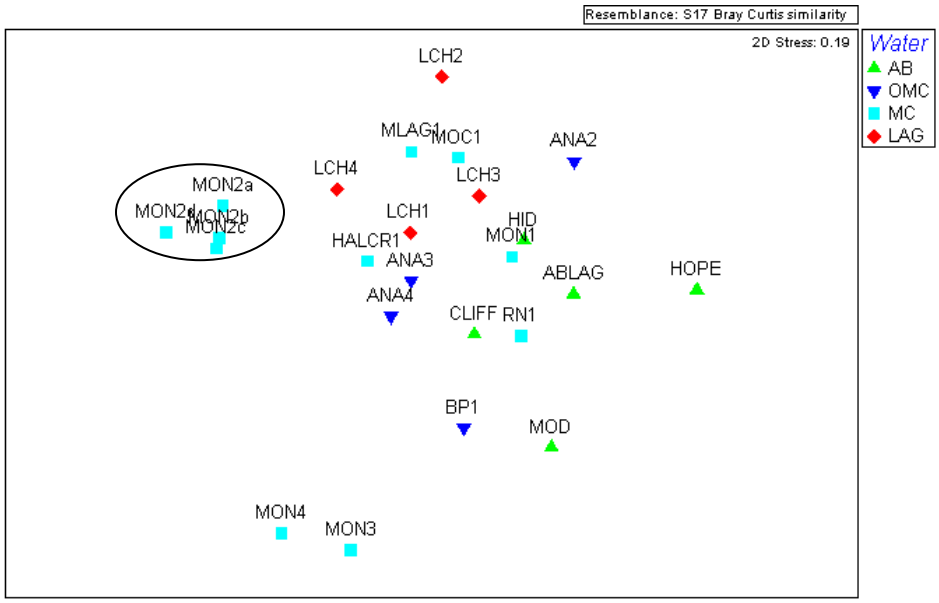

FIGURE 4-13: MDS plot showing group 3, Fig. 12, only. The MON 2a – 2d samples are indicated in a circle. The symbols grouped samples according to watershed/creek, with groups Arroyo Burro (AB), Mission Creek (MC), Old Mission Creek (OMC) and Laguna Channel (LAG).

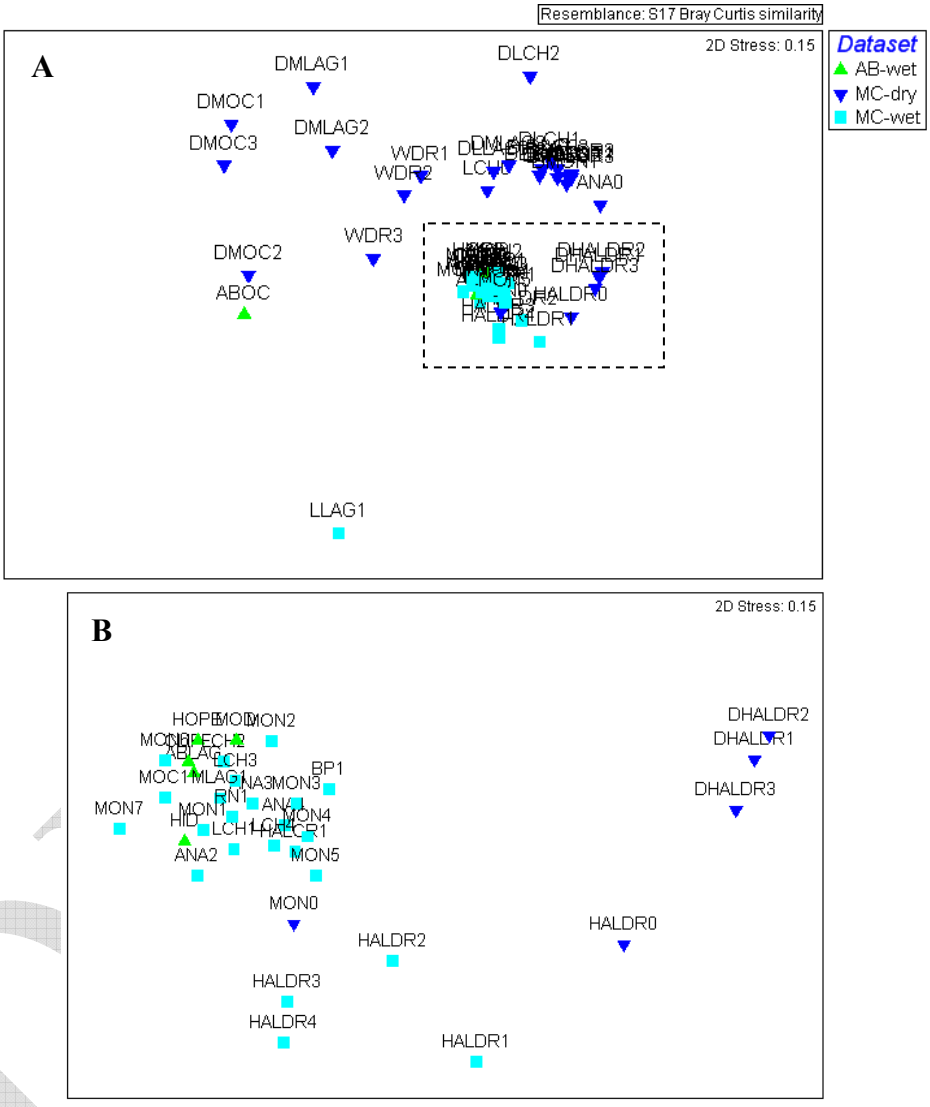

FIGURE 4-14: MDS plot showing grouping of combined dry and wet weather normalized TRFLP peak heights. The symbols grouped samples according to the dataset, with groups Arroyo Burro wet weather (AB-wet), Mission Creek dry weather (MC-dry), Mission Creek wet weather (MC-wet). A. All data, B. zoom of dashed area

Chapter 5: Source-Water Dependent Growth of Non-Target Bacteria in Colilert and Enterolert Fecal Indicator Assays (Phase II Clone Library analysis)

(submitted for publication to Applied and Environmental Microbiology)

5.1 Introduction

The detection and quantification of fecal pollution in recreational waters is of primary importance for protecting the health of swimmers. The standards for recreational water microbiological quality are based on culturable fecal indicator bacteria (FIB), i.e. total coliforms, fecal coliforms or *Escherichia coli*, and enterococci (49). These indicators themselves are not necessarily pathogenic, but they are commonly associated with pathogens (51). Various methods are accepted for quantifying FIB, including multiple tube fermentation, membrane filtration and define substrate technologies (DSTs) (13, 17, 68). All of these methods have in common the use of temperature and substrate conditions that are meant to be selective for the target organisms, but they differ in the exact substrates used. The DST to detect total coliforms and *E. coli* was originally developed for application in drinking water in the late '80s (23), but has since then been tested for its use in marine water (21, 33, 63, 65) and freshwater (12, 14, 15, 19, 26, 65), in different climates. The DST for detection of enterococci was developed in the early '90s (30, 40). Recently, the commercially available DST-based Colilert and Enterolert assays (IDEXX Laboratories, Westbrook, Maine) have been accepted by the U.S. Environmental Protection Agency as alternatives to the classical multiple tube fermentation and membrane filtration for fresh, marine or estuarine surface waters (75).

The Colilert assay relies on β-galactosidase cleavage of *o*-nitrophenyl-β-galactopyranoside to form a yellow product (*o*-nitrophenol) in detecting total coliforms. The detection of *E. coli* relies on the additional production of fluorescence, by β-glucoronidase cleavage of 4-methylumbelliferyl-β-D-glucuronide to form 4-methylumbelliferone. Hence, the simultaneous detection of a yellow color and fluorescence indicates the presence of *E. coli*. Enterolert targets the enzyme β-glucosidase to produce 4-methylumbelliferone from 4-methylumbelliferyl-β-D-glucoside, rendering an initially yellow substrate fluorescent. The enzyme-substrate relationships

that are the basis for these assays are expected to increase their specificity (68), and the Colilert and Enterolert methods are attractive because they require less manipulation and quality control compared with the classical multiple tube fermentation and membrane filter techniques (27, 68). Yet there has been evidence that these DST assays are not specific.

False-positive Colilert or Enterolert readings can occur, in case non-target bacteria possess the enzymes necessary to cleave the chromogenic/fluorogenic substrate while being present in sufficient concentrations after the assay's incubation period, so that the color/fluorescence can be detected. The latter can be accomplished by growth of the non-target bacteria during incubation, or by their high initial concentration in the samples. β-Galactosidase activity has been described for non-target species in the genera *Aeromonas*, *Pseudomonas*, or *Flavobacterium* (19, 25). β-Glucoronidase activity has been found in *Shigella*, *Salmonella* and *Yersinia* strains, *Flavobacteria* and in some streptococci, clostridia, *Bacteroides* spp. and *Corynebacterium* spp. (19, 58, 68). Most of these taxa don't grow using the added substrates, thus large numbers are required (>10^4 - 10^5 ml^{-1}) to yield false-positive results (19, 27). *Aeromonas* spp. concentrations in freshwater are lower than this threshold, even during sewer overflows or rainfall (28, 39, 41). However, *Pseudomonas* and *Flavobacterium* spp. concentrations reported in freshwater are very variable, and can reach the 10^4 - 10^5 ml^{-1} range (22, 35, 45, 50). Also, low concentrations of *Shigella* spp. (10 - 100 l^{-1}) can induce positive total coliform and *E. coli* responses in freshwater (9), implying they grow using the DST test substrates. "False-positive" responses from *Shigella* spp. would offer increased protection for the public health, since low concentrations of this bacterium can cause bacterial dysentery (9). In the Enterolert assay, several non-target bacteria were identified that yielded false-positive scores, such as *Proteus vulgaris*, *Serratia marcescens*, *Sphingomonas* spp. and *Flavobacterium* sp. (2, 13). However, non-enterococcal bacterial concentrations up to 2 x 10^3 ml^{-1} did not cause a fluorescent signal (16), again indicating that these bacteria don't grow in the DST medium. Overall, these previous studies also suggest that the Colilert test specificity may vary according to the nature of the water sample. On the other hand, comparatively little knowledge is available about the occurrence of false-positives in the Enterolert assay. While all this prior evidence provides insights into the growth of

isolatable non-target organisms, culture independent analysis of Colilert and Enterolert enrichments could more fully reveal the extent of non-specific growth.

This research aims at identifying the bacteria that grow in Colilert and Enterolert enrichments of different water samples. We hypothesized that the occurrence of false-positives in Colilert/Enterolert tests depends on the bacterial community composition of the water samples. The idea is also supported by recent literature in microbial endemism, suggesting that aquatic microbial communities retain their phylogenetic distinctions, even when differently-inoculated enrichments are conducted over time frames that are sufficiently long enough for rare populations to increase (54). To test our hypothesis, we used culture-independent techniques (clone library and terminal restriction length polymorphism analysis) to analyze the bacterial diversity in different source water samples: two urban creek water samples (one before and one during a rainstorm) and one sewage influent sample. Colilert and Enterolert tests were used to determine the concentration of fecal indicator bacteria in these samples, and the bacterial diversity in the yellow/fluorescent and fluorescent wells, respectively, were analyzed using the same culture-independent techniques. Bacteria growing in the Quantitray/2000 assays will be represented by dominant clones or terminal restriction fragments.

5.2 Materials and Methods

5.2.1 Study sites and Sampling

Arroyo Burro Creek is a 303d-listed (for pathogens) creek that drains a semi-urban watershed in Santa Barbara, CA. The Arroyo Burro watershed is 25.6 km^2 with 27% residential, 10% commercial, 61% open, and 2% agricultural space. The creek enters into a brackish lagoon that terminates at a popular beach, frequently posted with warnings due to high fecal indicator organism concentrations. We sampled Arroyo Burro Creek just upstream of the discharge in the lagoon on October 24th, 2005 (DRY), at the end of the dry season and on November 9th, 2005 (WET), during the first winter storm. One sample was also taken from the raw sewage influent of El Estero wastewater treatment plant (SEW) on October 24th, 2005. Two liter water samples were collected approximately 10 cm beneath the surface and immediately passed through Miracloth

(Calbiochem-Novabiochem, La Jolla, CA) to remove large debris and stored on ice until further processing in the lab (maximum 6 hours).

Total coliforms, *E. coli* and enterococci were quantified using the Colilert and Enterolert Quanti-Tray/2000 (IDEXX Laboratories, Westbrook, Maine, USA) per manufacturer's instructions. All yellow/fluorescent Colilert (presumed *E. coli*-positive) or fluorescent Enterolert (presumed enterococci-positive) wells of each assay were pooled before DNA extraction, and these samples were indicated using the suffix –C and –E, respectively (e.g. DRY-C and DRY-E).

5.2.2 DNA Extraction

DNA was obtained from the source water samples using the UltraClean Water DNA Kit (MoBio, Carlsbad, CA, USA), according to manufacturer's instructions. In short, the samples were filtered under vacuum through 0.22 μm filters to collect the bacteria, and the filters were frozen until further use. Filtration was to the point of filter refusal, and thus the filtered water volumes differed: 1630 ml (DRY), 430 ml (WET) and 135 ml (SEW). After extraction, the DNA was ethanol precipitated and redissolved in 50 μl sterile Nanopure water. DNA was also extracted from the medium from yellow/fluorescent Colilert and fluorescent Enterolert wells, obtained by piercing a sterile syringe through the sterilized (70% ethanol swabbed) paper backing of the plastic Quantitray/2000. The well contents were pooled into single within-tray samples. DNA of the pooled samples (28 - 43 ml) was extracted using the UltraClean Water DNA Kit, as described above. Final DNA concentrations were determined with fluorometry, using the Quant-iT Picogreen dsDNA Assay Kit (Molecular Probes, Eugene, OR, USA), according to manufacturer's instructions.

5.2.3 Terminal Restriction Fragment Length Polymorphism Analysis

PCR and terminal restriction fragment length polymorphism (TRFLP) analysis were based on a previously published method (18). Briefly, genes encoding bacterial 16S rRNA were PCR amplified using the primers 8F hex (fluorescently labeled forward

primer; 5'AGAGTTTGATCCTGGCTCAG (56)) and 1389R (5'ACGGGCGGTGTGTACAAG (62)) as described before (53). PCR products were purified with a commercially available kit (QIAGEN, Valencia, CA), and ca. 300 ng of purified DNA was digested with *Hha*I (New England Biolabs, Ipswich, MA, USA). After inactivation of the restriction enzyme by heating (65°C, 20 min), the lengths of fluorescently labeled fragments were determined with an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the Genomics Technology Support Facility (Michigan State University). Only terminal restriction fragments (TRFs) having lengths between 50 and 1000 bp could be reliably analyzed. TRFs, based on peak heights, were aligned and normalized (66), discarding peaks with a relative peak height less than 1%. The automatic alignment was checked manually, resulting in the final assignment of TRF length 215 to the TRF 215.56 in SEW-E (instead of 216). All other alignments were maintained.

The *in silico* PCR and Restriction program of the web-based tool Microbial Community Analysis (MiCA) (71) was used to list the possible phylogenetic affiliations of the major peaks from our electropherograms. A size window of ± 3 was used to account for the possible differences between real and predicted TRF lengths (59, 61). Only taxa that were found in the clone library analysis were retained in the *in silico* digestion. The Primer 6 software (PRIMER-E Ltd) was used to calculate diversity indices and perform non-metric multidimensional scaling (MDS), based on normalized peak heights in the TRFLP profiles. Resemblances were calculated using the Bray-Curtis similarity coefficient and MDS was subsequently performed using 100 restarts, Kruskal fit scheme 1 and a minimal stress of 0.01. The SIMPROF permutation test in Primer 6 was used to look for statistically significant evidence of genuine clusters in the dataset (at a significance level of 0.05). It is a permutation test of the null hypothesis that a specified set of samples, which are not a priori divided into groups, do not differ from each other in multivariate structure.

5.2.4 Clone Library Analysis

To generate a clone library, partial 16S rRNA genes were amplified from purified DNA from all samples. The primers 8F (no fluorescent label) and 1398R were used as above and PCR products were cloned into the pCR®2.1 vector (Invitrogen, Carlsbad CA). The vectors were transformed into *E. coli* and grown overnight on LB plates containing 50 μg ml^{-1} ampicillin. Blue/white screening and further processing and sequencing were performed by Agencourt Bioscience (Beverly, MA, USA). The primers used for sequencing were the same as for PCR amplifications described above (8F/1389R). Sequencing at Agencourt Bioscience was performed using the BigDye™ Terminator Version 3.1 chemistry, which is optimized for longer reads, uniform peak heights and signal. However, the sequence close to the primer is lost, and this compromised the specific prediction of TRFs from our clone sequences. Therefore, relating the clone library and TRFLP results was done using *in silico* digestion, as described above.

Clone sequences were manipulated using the BioEdit sequence alignment editor (38), that includes the ClustalW Multiple alignment module (73). Alignments were manually checked and corrected if necessary. Nucleotide positions with gaps were also deleted (7). The occurrence of chimeras was determined using Bellerophon (43), Check_Chimera (57), Pintail (5) and manual comparison of putative chimeric sequences. Between 0 and 27 chimeras (12 on average) were identified for each 96 clones in our 9 clone libraries. Based on the sequence identity matrix, clones sharing ≥ 97% identity were grouped into one operational taxonomical unit (OTU). The BLAST algorithm (3) was used to determine the phylogenetic affiliations of all OTUs. Phylogenetic neighbor joining trees showing OTUs and reference strains were constructed using PAUP* 4.0 (Sinauer Associates, Inc., Sunderland, MA), using 1000 replicates for bootstrap analysis. Rarefaction analysis and estimation of the clone library richness were performed using a web interface (http://www.aslo.org/lomethods/free/2004/0114a.html) (46).

5.2.5 Nucleotide Sequence Accession Numbers

The nucleotide sequences obtained in this study have been deposited in Genbank under accession numbers EF658766-EF659414.

5.3 Results

5.3.1 Creek Water Characteristics and IDEXX Results

The sample DRY was taken during dry weather, and no precipitation had fallen within the previous 7 months. During the first winter storm in the area on 11/09/05, the Creek flow rate increased sharply from base flow (46 m^3 h^{-1}) at 03:30 to a storm flow rate of ~ 13 – 19 x 10^3 m^3 h^{-1} between 7:30 and 9:00. Sample WET was taken that day at 7:30, at a flow rate of 13 x 10^3 m^3 h^{-1}. The Creek water temperatures remained between 12.5ºC and 17.2ºC during this whole period.

Table 5-1 presents the results of the IDEXX assays for the 3 samples. The raw sewage sample contained the highest number of FIB. Also, FIB concentrations (*E. coli* and *Enterococcus*) in the creek increased 80- to 148-fold after the storm.

5.3.2 TRFLP Analysis

The TRFLP fingerprints (Fig. 5-1), indicate a comparatively diverse pattern for the source water samples, but a limited number of peaks for the pooled yellow/fluorescent Colilert (YF-C) and fluorescent Enterolert (F-E) enrichments. Also, differences are obvious between samples in a particular enrichment (e.g. DRY-C vs. SEW-C, DRY-E vs. WET-E). The TRF lengths of the most abundant peaks (> 50 bp, see materials and methods) in the enrichments are indicated, together with the putative phylogenetic affiliation, based on *in silico* digestion (see results below). For the source water samples, the samples WET and SEW shared more peaks (86, 98, 202, 205 and 565) than did DRY with either WET (86, 365 and 570) or SEW (86). Only one TRF, of 86 base pairs (bp), was shared by all source water samples. In the YF-C enrichments, the TRFs of 210 and 569 bp were shared by all samples. Also the peaks 370, 371 and 576 were shared by 2 out of 3 YF-C enrichments. All F-E enrichments shared TRFs with

lengths 215 and 593 bp. A few peaks were shared between the TRFLP patterns of the source water samples and the YF-C or F-E enrichments. The small peaks (both ~2% relative peak height) with length 210 (in WET) and 569 (in SEW) were present in all YF-C enrichments, with relative peak heights in the latter of 72% and 6%, respectively. Also, TRF peak 202 had 13 – 16% relative peak intensity in WET and SEW, and was a minor peak in WET-E (1.4%).

The similarities between all TRFLP patterns, based on normalized peak heights, are shown using an MDS plot (Fig. 5-2). A SIMPROF test ($\alpha = 0.05$) indicated that some grouping of the samples occurred according to sample type (source water vs. YF-C enrichment vs. F-E enrichment), but the inclusion of SEW-C and WET-E was not statistically significant. The average within-group sample similarities were smaller for the source water samples (16%) than for the YF-C (30%) and F-E (41%) samples.

5.3.3 Clone Library Composition of Source Water Samples

The bacterial diversity in the different source water samples, at phylum or class level, is shown in Fig. 5-3A. In all samples, *Bacteroidetes* (phylum) and β-*Proteobacteria* were the most abundant groups. The raw sewage influent differed from the creek water samples by the large representation of ε-*Proteobacteria*, *Fusobacteria*, and to a lesser extent *Firmicutes* and γ-*Proteobacteria*. The Creek water samples differed by the higher number of phylogenetic groups detected in WET (groups that are indicated with * in Fig. 5-3A).

A detailed representation of the bacterial diversity of the source water samples is shown using two phylogenetic trees, one for all *Bacteroidetes* (Fig. 5-4A) and one for all β-*Proteobacteria* (Fig. 5-4B). All *Bacteroidetes* clones from SEW clustered separately from the other *Bacteroidetes* clones in two clusters, indicated in Fig. 5-4A. Cluster SEWa contained clones that belonged to the *Flavobacteriaceae* family. Cluster SEWb contained clones from the genus *Bacteroides*, a genus that was exclusively found in the SEW clone library. Also a WET-specific cluster appeared (WETa), including *Sphingobacteriales* clones. The remaining clones from DRY and WET mostly belonged to the *Flavobacteriales*. The clones belonging to the β-*Proteobacteria* were largely represented

by members of the order *Burkholderiales*, and especially the *Comamonadaceae* family. Moreover, the DRY library only consisted of *Comamonadaceae* clones, and were almost all separated in cluster DRYa. A large share of the clones from WET, were grouped separately (WETb in Fig. 5-4B). Besides the *Comamonadaceae*, the *Oxalobacteriaceae* and *Alcaligenaceae* (clone SEW-A09) families of the *Burkholderiales* were also represented in the creek water clone libraries. Other orders represented were the *Methylophilales*, *Neisseriales* and *Rhodocyclales*. The clones not belonging to the *Bacteroidetes* or β-*Proteobacteria* were assigned to a variety of phyla, some of which are exclusive to the WET library (phylogenetic tree not shown, see also Fig. 5-3A).

We analyzed the origin of all clones/isolates that showed the highest percentage identity with the clones from our clone libraries. These clones were also included as reference strains in the phylogenetic trees. Remarkable differences in origin were found for the 3 source water libraries. For DRY, the majority of the clones (69%) were most closely related with clones/isolates obtained from freshwater (rivers or lakes). Only 15% were related with sources such as soils, sediments, activated sludge, biofilms and groundwater and the remaining 16% with salt water samples. Also the phylogenetic tree (Fig. 5-4B) shows that most clones from DRY (36 out of 62) were closely related with the cluster DRYa, grouping with clones/isolates from freshwater environments. Conversely, for WET only 11% of the clones were most related with clones/isolates originating from freshwater. The majority (72%) was related with clones/isolates derived from soils, sediments, activated sludge, biofilms, microbial mats and groundwater. The remainder of the clones was related with other environments (e.g. oceans, glaciers) or no consistent source environment could be assigned. For the raw sewage influent (SEW), 41% of the clones were most closely related with clones/isolates derived from sludge, intestine or fecal samples. An additional 21% were most closely related with clones from mangrove bacterioplankton, but also very closely related with swine fecal material. The remaining 38% of the clones were not affiliated with sewage-related materials, but with groundwater, iron deposits, sediments and to a smaller extent freshwater(-biofilm) and oil.

5.3.4 Clone Library Composition of YF-C and F-E Enrichments

The composition of the clone libraries of the YF-C enrichments is summarized in Fig. 5-3B. While all clones from SEW-C belonged to the *Enterobacteriaceae*, and the majority (67%) was most closely related with *E. coli*, this was not the case for the creek water sample enrichments. In DRY-C, 73% of the clones belonged to the genus *Vibrio* and 1% to the genus *Shewanella*. In WET-C, none of the clones was identified as *E. coli*. Again, most of the clones were not assigned to the *Enterobacteriaceae*, but to *Vibrio* (47%) and *Shewanella* (27%).

A summary of the phylogenetic affiliations of the clones from the F-E enrichments is shown in Fig. 5-3C. For DRY-E and SEW-E, all clones were assigned to the target genus, i.e. *Enterococcus*. However, for WET-E, a large percentage of the clones were non-enterococci, belonging to the genus *Clostridium* (7%), the phylum *Bacteroidetes* (15%) or the β-*Proteobacteria* (3%). Unfortunately 50% of the clones, all grouped in the same OTU, could not be identified beyond the bacteria level. There were no indications these clone sequences were the results of chimera formation, and all were most closely related (92 – 99% identity) to an uncultured bacterium (Genbank accession nr. AB269520) from solid waste compost.

5.3.5 TRFLP Peak Identification of YF-C and F-E Enrichments

The direct prediction of TRFs from the clone sequences in the database was compromised due to the sequencing chemistry used (see Materials and Methods); instead we predicted the bacterial species that corresponds with the major TRFLP peaks of the pooled Quantitray/2000 enrichments using the MiCA web tool. From all genera predicted using the *in silico* digestion, only those genera that belonged to taxonomic groups found in the enrichment clone libraries were considered relevant to this study, other genera were not included. These taxonomic groups are, for YF-C: *Enterobacteriaceae*, *Comamonadaceae*, *Vibrio* spp. and *Shewanella* spp., for F-E: *Enterococcus* spp., *Bacteroidetes*, *Clostridium* spp. and *Burkholderiales*. Note that the γ-, δ-*Proteobacteria* and *Acinetobacter* were not included for YF-C because they only represented one clone each (~1.5% of total clones). The phylogenetic affiliations of the major peaks from the

enrichments are shown in a separate box in Fig. 5-1. We didn't report the *in silico* digestion for the source water samples, since many TRFLP peaks could usually be assigned to various genera that all were present in the clone library. Hence, reliable peak identification was not possible in this case.

In the YF-C enrichments, the major TRF for both creek samples had a length of 210 bp. The most likely genus predicted by MiCA, consistent with the clone library, was *Vibrio*, and more precisely *Vibrio cholerae* or *Vibrio mimicus*. All other *Vibrio* spp. were represented by other TRFs, e.g. 383 bp for *Vibrio parahaemolyticus*. The TRFs of 370 – 372 bp (corresponding with several *Enterobacteriaceae*) were present in all samples, but only dominant in the SEW-C sample, as was similarly indicated by the clone library. The TRF of 576 bp, being dominant in WET-C, was assigned to *Shewanella* spp., which had also a high incidence in the clone library of that sample. The identity of the minor peak of 569 bp could not be unequivocally determined, since the peak could belong to *Shewanella* spp. or members of the family *Comamonadaceae*.

The F-E enrichments all had a major TRFLP peak at 215 bp, which was attributed to *Enterococcus*. In WET-E, where significant growth of non-enterococci (*Bacteroidetes*) was observed using the clone library, the dominant TRFLP peak had a length of 99 bp. This peak was affiliated with a number of *Bacteroidetes* genera, but not with enterococci or clostridia. The TRFs with lengths 153 and 229 bp could be assigned to *Burkholderiales* and *Clostridium* spp., respectively. Peak 593, occurring in all F-E enrichments, could be related with a number of *Lactobacillales* or *Clostridiales* genera (although no *Clostridium* spp.). The peaks 153 and 593 indicated the presence of non-enterococci in DRY-E and SEW-E, which were probably minority populations overlooked by the clone library.

5.3.6 Bacterial Diversity in Source Waters and Enrichments

Overall bacterial diversity parameters for the source water samples were calculated in two ways. First, diversity indices (Shannon-Weaver diversity, H and Species Richness, S) were calculated using the normalized peak heights from the TRFLP analysis. Second, rarefaction analysis of the clone libraries was used to determine the

Chao richness estimator (S_{Chao}). The results are summarized in Table 5-2. All diversity indicators indicated a strong increase in bacterial diversity in Arroyo Burro creek during a storm (WET) compared with dry weather conditions (DRY). The bacterial diversity in the sewage influent was intermediate between both Creek water samples.

5.4 Discussion

Fecal indicator assays are a requisite component of microbiological water quality monitoring. Yet for FIB assays to hold value, they should equivalently, across a range of source water samples, reflect the possible presence of pathogens and hence risks to human health. While, for the purpose of this study, we accept the concept that FIB concentrations reflect the presence of pathogens, we do argue that positive signals in the Quantitray/2000 assays are not always caused by the target fecal indicator bacteria. Prior studies have hinted, from phenotypic profiling of enrichment isolates, that FIB assays can encourage the growth of non-target organisms and that this outcome varies by sample (1, 13, 15, 19, 25, 26, 30, 63, 65). Here, through culture-independent microbial analysis of source waters and FIB enrichments (Colilert and Enterolert), we further define the degree of this problem, and show that the growth and color/fluorescence production of non-target organisms likely causes the FIB assays to overestimate the risk for human health, depending on the source water sample. While a higher bacterial diversity in the source water samples correlated with a higher bacterial diversity in the YF-C and F-E enrichments, the former was not unequivocally related with the growth of non-target organisms.

First, we determined how the bacterial communities from the 3 source samples differed, using TRFLP and clone library analysis. Rarefaction analysis indicated that most clone libraries represented the diversity in their source environment well, except for samples WET, WET-C and SEW (Table 5-2). In the latter cases, the S_{Chao} did not reach a stable asymptotic value, indicating an underestimation of the real diversity. The TRFLP-based diversity indicators cannot express total bacterial diversity; in this case they are used to compare diversity between the different samples. The species richness found here was similar as in previous studies (using *Hha*I) for freshwater or sewage samples (29, 52). More importantly, all diversity indices indicated that the bacterial diversity differed

greatly among samples. Moreover, the bacterial community composition in the creek water seemed related to the occurrence of precipitation. During dry weather, most clones from the creek water belonged to the β-*Proteobacteria*, *Bacteroidetes* and *Actinobacteria*, which is consistent with previous reports on freshwater bacterioplankton diversity (4, 10, 20, 69, 79). The majority of the clones from the same sample were also most closely affiliated with clones/isolates from freshwater environments, and were members of well-known freshwater clusters *Rhodoferax* sp. BAL47 (β-*Proteobacteria*; (79)) and acII-B (*Actinobacteria*; (76)), even more so indicating that the creek water during dry weather can be characterized as a typical freshwater habitat. Conversely, during the storm the bacterial diversity in the creek increased drastically, and only 11% of the clones from this water sample were putative freshwater clones. Most clones seemed to be derived from environments such as soils, sediments, activated sludge, biofilms, microbial mats and groundwater. While it has frequently been observed that rainstorms and runoff change the bacterial communities in freshwater, leading to increases of total (44) or fecal indicator (37, 67, 70, 72) bacterial numbers, this study provided more detail about the qualitative changes in bacterial community composition. The relative proportions of non-freshwater bacteria increased during precipitation, most likely following the input of allochtonous bacteria from various sources in the watershed (20, 52, 72). It has been found that bacterial communities can also change due to the input of growth-stimulating nutrients (32, 36, 42, 77), but it's unlikely that this occurred here, as the nutrient concentrations (nitrate, ammonium and phosphate) were not affected by the precipitation and subsequent flow rate increase (results not shown). While the bacterial community compositions in our source water samples were quite distinct, overall they were similar to what we might expect from prior reports. However, the communities that developed in the FIB enrichments of some samples were somewhat surprising.

The clone library analysis allowed a detailed analysis of the taxa present in the FIB enrichments. In the YF-C enrichments of both creek water samples, only a small part of the clones were identified as *E. coli* or as a member of the coliform group of the *Enterobacteriacae* (*Escherichia*, *Klebsiella*, *Enterobacter* and *Citrobacter* (19, 34, 74)). The majority of the clones belonged to the non-target genera *Vibrio* (in DRY-C and WET-C) and *Shewanella* (in WET-C), as was observed using TRFLP. Also, clones

assigned to the non-coliform *Enterobacteriacae* genera *Kluyvera*, *Shigella*, *Providencia* and *Plesiomonas* were present. Our data contrast greatly with the claim that the Colilert assay specifically grows coliforms, with chromogene production and *E. coli*, with fluorochrome production (31). The simultaneous detection of *E. coli* together with other coliforms in yellow/fluorescent Colilert wells has been observed before (15), and was also expected, as total coliform numbers exceeded *E. coli* readings by a factor 8 – 50. The detection of non-target bacteria in yellow/fluorescent Colilert tubes/wells has also been observed before, for instance in freshwater, where *Salmonella* spp. were isolated, although only in a minority of the samples (19, 65). In tropical freshwater, non-coliforms such as *Rahnella*, *Aeromonas*/*Vibrio*, *Pantoea*, *Salmonella*/*Shigella*, *Serratia*, *Kluyvera*, *Providencia*, *Aeromonas* and *Shewanella* (in decreasing order of importance) have been isolated (15). While all clones from the creek water YF-C samples belonged to genera isolated before in yellow/fluorescent Colilert wells from freshwater samples, the dominance of *Vibrio* and *Shewanella* clones was not expected based on these previous isolation studies. Furthermore, the recovery of vibrios from yellow/fluorescent Colilert wells is usually associated with marine water samples (63, 65). The increased detection of *Vibrio* spp. (and to a lesser extent *Shewanella* spp.) in the YF-C samples in this study, compared with other freshwater studies, is likely related with the use of culture-independent techniques. Previous culture-based studies used culture media selective for coliforms, such as MacConkey, m-ENDO and m-TEC agar, to isolate bacteria from yellow/fluorescent Colilert wells/tubes. The importance of the culture medium was exemplified in a previous study, in which vibrios were only isolated from yellow/fluorescent Colilert wells (from marine water samples) using thiosulfate-citrate-bile salts-sucrose (TCBS) medium, but not using MacConkey agar (63). The reason for the dominance of *Vibrio* spp. in our YF-C enrichments samples might be the competitive advantage they have because of a reduced grazing pressure during enrichment. Bacteria forming large cells (e.g. *Alteromonas* and *Vibrio* spp.) are vulnerable to grazing, explaining their rarity in the water column. When reduced grazing pressure is artificially reduced (e.g. by dilution), these rare bacteria rapidly overgrow the original microbial assemblages (8, 64). As culture-independent techniques showed that non-coliforms were dominant in the YF-C enrichments, it was important to determine whether these non-

target bacteria could cause a yellow color or fluorescence. From all of the genera detected (except *Shewanella*), at least one species has shown to yield a yellow color in the Colilert assay (15, 21, 63). However, besides *E. coli*, production of fluorescence in the Colilert test was only suggested for *Vibrio alginolyticus* (65), but not for *Vibrio cholerae* or *Vibrio mimicus*, species found in this study. Therefore, we think it is unlikely that the non-target bacteria growing in the Colilert assay produced the fluorescent signal. They probably co-exist in the wells with *E. coli*, the latter causing the fluorescence. The clone library and TRFLP analysis confirmed the previous studies indicating that false-positive signals for total coliforms (only production of yellow color) are very likely to occur in the creek water samples, due to growth of *Vibrio* spp. and non-coliform *Enterobacteriaceae*. Also, this study provided the first report of *Shewanella* spp. growing in the Colilert assay, although it hasn't been shown that the bacterium can cause a false-positive total coliform or *E. coli* reading.

The specificity of the Enterolert assay, on the other hand, was confirmed by clone library analysis for 2 out of our 3 samples (DRY-E and SEW-E). Only in WET-E were a significant number of non-target clones (*Bacteroidetes*, *Clostridium* spp. and *Burkholderiaceae*) present. Also, the dominance of an unknown bacterial clone (partly alignable with a *Bacteroidetes* isolate) increases the extent of non-specific growth. These results contrast slightly with the TRFLP results, which also indicate growth of *Lactobacillales*/*Clostridiales* in DRY-E and SEW-E, and *Burkholderiales* in SEW-E, with relative peak heights between 5% and 11%. Previous studies have indicated that the number of false positives for the Enterolert assay in recreational waters is generally low, between 2.4-5.1% (1, 13, 30, 40). None of these previous studies suggested the extensive growth of non-enterococci as observed in WET-E. While the growth of *Flavobacteria* (but no other *Bacteroidetes* bacteria) and *Lactobacillales* in the Enterolert medium was shown before (13), this was not the case for clostridia or *Burkholderiales*. Other bacteria that have been isolated before from fluorescent Enterolert wells, but were not found in this study, include *Bacillus* spp., *Proteus vulgaris*, *Serratia marcescens*, *Sphingomonas paucimobilis*, *Providencia stuartii*, *Roseomonas fauriae*, *Vibrio parahaemolyticus* and *Pasteurella multocida* (1, 13, 30). Hence, there is a discrepancy between our results and previous reports. As with the Colilert enrichments, it is likely that the use of culture-

independent techniques in this study provides a different view of the non-target bacteria growing in fluorescent Enterolert wells. The culture media that have been used before for isolation and subsequent phenotypic profiling of bacteria growing in fluorescent Enterolert wells included bile-esculine agar (1, 13) (used to differentiate enterococci and group D streptococci, but also allows growth of some *Enterobacteriaceae* (55)) and MacConkey agar (30) (grows coliforms). Thus, it is highly unlikely that clostridia or *Burkholderiales* would have been detected using these media and (aerobic) incubation conditions. Moreover, selective enrichment of minority populations from the Quantitray/2000 wells probably also occurred in the growth media used by others. It hasn't been shown before, using Enterolert assays, that clostridia and *Burkholderiales* can cause false-positive fluorescent signals, although this was shown for some other non-target bacteria (*Proteus vulgaris*, *Serratia marcescens*, *Sphingomonas* spp. and *Flavobacterium* sp. (2, 13)), including *Bacteroidetes*. However, all of the bacterial groups detected in the F-E enrichments contain species with significant β-glucosidase activity (6, 11, 47, 48, 60), which makes it possible that they cause false-positive Enterolert readings. Since the enzyme activity can differ substantially, even within genus or species (60), direct proof of the occurrence of false positives should involve isolation of bacteria, which was not performed in this study. Also, most *Bacteroidetes* identified in WET-E belonged to the class *Sphingobacteria* (see discussion below and Fig. 5-6) while β-glucosidase activity has only been observed for the class *Bacteroidetes* (6, 11, 60). Nonetheless, the results obtained here imply that the risk for human health may be overestimated by using the Enterolert assay, depending on the water sample, due to the growth and fluorescence of non-target bacteria.

The non-culture based techniques used in this study indicated extensive growth of non-target bacteria in YF-C and F-E enrichments. Furthermore, based on previous studies, we can infer that these non-target bacteria are likely to cause false-positive results, because of their color or fluorescence production. However, this extent of non-specific growth did not occur in all samples. The bacterial community in the raw sewage (SEW-C) sample, was dominated by *E. coli* clones, and most other clones were coliforms, conform to what was expected. Also, in the DRY-E and SEW-E samples, only enterococci clones were present, although small peaks belonging to other taxa were

present in their TRFLP patterns. The SIMPROF permutation test indicated that SEW-C (in Colilert) and WET-E (in Enterolert), differed in multivariate structure (i.e. bacterial community composition) from the other samples in the same enrichment (Fig. 5-2). Hence, our results show that the extent of growth of non-target bacteria in Quantitray/2000 assays can change within weeks at the same sample location, e.g. after precipitation, and not only depends on general sample characteristics such as marine water vs. freshwater, sewage effluent vs. freshwater or temperate vs. (sub)tropical freshwater, as observed before (14, 15, 24-26, 65, 78). Evidently, this may have important implications when monitoring FIB concentrations in freshwater bodies throughout the year, as has been done in studies at several locations in the U.S. (37, 49, 67).

We investigated if there was a relation between the bacterial community composition in the source water, and the growth of non-target organisms in the FIB assays, as had been suggested before (25, 78). Firstly, our data showed that an increased bacterial diversity in the source water was related with increased diversity in the FIB enrichments (see Table 5-2), with R^2 values between 0.68 – 1.00 for all diversity estimators. Secondly, in the F-E samples, we observed positive a relation between the proportion of non-target bacteria and the source water bacterial diversity: non-target bacteria were only found in WET-E (75%), while the WET source water sample had the highest bacterial diversity. However, this was not the case for the YF-C samples, in which the proportion of non-target bacteria was similar in DRY-C and WET-C, but lower in SEW-C. One previous study showed that wastewater effluent, with a higher bacterial diversity, produced a higher number of false positives in Colilert and Enterolert assays than freshwater, having a lower bacterial diversity (78). Based on our results, we conclude that a higher bacterial diversity in the source water sample causes a higher bacterial diversity in the FIB enrichments, although this does not automatically lead to a higher number of false positives. However, to obtain statistically relevant relationships between source water bacterial diversity and the growth of non-target bacteria in FIB enrichments, a more extensive dataset should be analyzed. Perhaps bacterial diversity is a too generalizing parameter for determining the potential of false-positive Quantitray/2000 results, and the presence of specific taxonomic groups in the source water, able to grow

in the FIB assays, might be a better predictor. If this were the case, a higher abundance of *Vibrio* spp., *Shewanella* spp., *Bacteroidetes* and clostridia in the source water samples should correspond to their increased detection in the FIB assays. However, this study showed no evidence for this, as *Vibrio* spp., *Shewanella* spp., and *Clostridium* spp. were not detected in the clone libraries of our source water samples. Although TRFLP analysis showed a TRF with length 210 in WET, we cannot infer that the peak also indicates the presence of *Vibrio* spp., similarly as in the YF-C samples. Other taxa, such as β-*Proteobacteria*, *Pseudomonas* and *Rhodobacteraceae*, present in WET, also produce TRFs in the range of 210 ± 3 bp. Phylogenetic analysis of all *Bacteroidetes* clones (not grouped according to OTU) in the source water samples and in WET-E showed that 9 out of 11 *Bacteroidetes* clones from WET-E formed a separate cluster (Fig. 5-5). This cluster only contained *Sphingobacterium* spp. (no reference sequences included in the phylogenetic tree). No other *Bacteroidetes* clones from the source water samples belonged to this genus, suggesting that especially *Spingobacterium* spp. are able to grow in the F-E enrichment. However, *Sphingobacterium* spp. were not detected in the source water samples.

5.5 Conclusions

Clone library and TRFLP analysis showed that that the proportion of non-target bacteria growing in yellow/fluorescent Colilert and fluorescent Enterolert wells varied depending on the nature of the source water sample. A more diverse bacterial community in the source water led to a more diverse community in the FIB enrichments, but not always to a higher proportion of non-target bacteria. For the Colilert assay, our results confirmed the potential of *Vibrio* spp. and non-coliform *Enterobacteriaceae* to cause false-positive total coliform readings, but indicated that *Shewanella* spp. may also bias Colilert results. In the Enterolert assay, false-positive results may be caused by *Bacteroidetes* (especially *Sphingobacterium* spp.), *Clostridium* spp. and *Burkholderiaceae* bacteria. These taxa were not known to grow in the Enterolert medium before. The clone library analysis finally showed that the rainstorm drastically increased the percentage of non-freshwater bacteria in the creeks.

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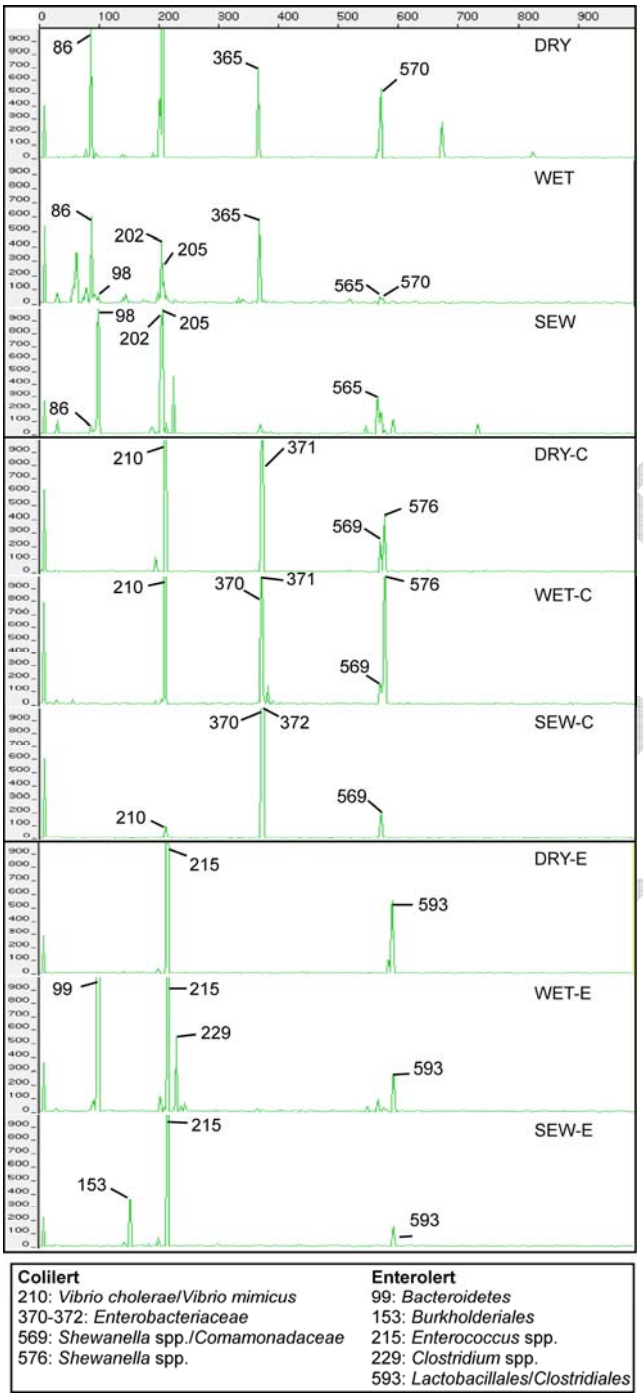
TABLE 5-1: Concentrations of fecal indicator bacteria from Colilert and Enterolert assays.

Sample	Total coliforms	*E. coli* (MPN 100 ml^{-1})	Enterococci
DRY	1.55×10^4	2.79×10^2	2.56×10^2
WET	3.45×10^5	4.12×10^4	2.05×10^4
SEW	2.76×10^8	1.15×10^7	1.48×10^6

TABLE 5-2: T-RFLP OTU richness (S) and Shannon Diversity (H), and clone library rarefaction-based Chao richness estimator (S_{Chao}) for source water samples and the Colilert (-C) and Enterolert (-E) enrichments.

Sample	Richness (S)	Diversity (H)	S_{Chao}
DRY	8	1.4	25
WET	22	2.7	567*
SEW	16	2.2	124*
DRY-C	5	0.9	9
WET-C	8	1.5	67*
SEW-C	5	1.1	10
DRY-E	3	0.4	7
WET-E	8	1.2	23
SEW-E	7	0.9	2

* Underestimated values based upon rarefaction analysis



Colilert	Enterolert
210: *Vibrio cholerae/Vibrio mimicus*	99: *Bacteroidetes*
370-372: *Enterobacteriaceae*	153: *Burkholderiales*
569: *Shewanella* spp./*Comamonadaceae*	215: *Enterococcus* spp.
576: *Shewanella* spp.	229: *Clostridium* spp.
	593: *Lactobacillales/Clostridiales*

FIGURE 5-1: TRFLP electropherograms for source water samples (upper box), yellow/fluorescent Colilert (middle box) and fluorescent Enterolert (lower box) enrichments. For the source water samples, peak lengths are shown for peaks shared between minimum two samples. For the Colilert and Enterolert enrichments, the most dominant TRF peak lengths are indicated. The putative phylogenetic affiliations assigned to the peaks from the Colilert and Enterolert enrichments, based on *in silico* digestion, are shown below the electropherograms.

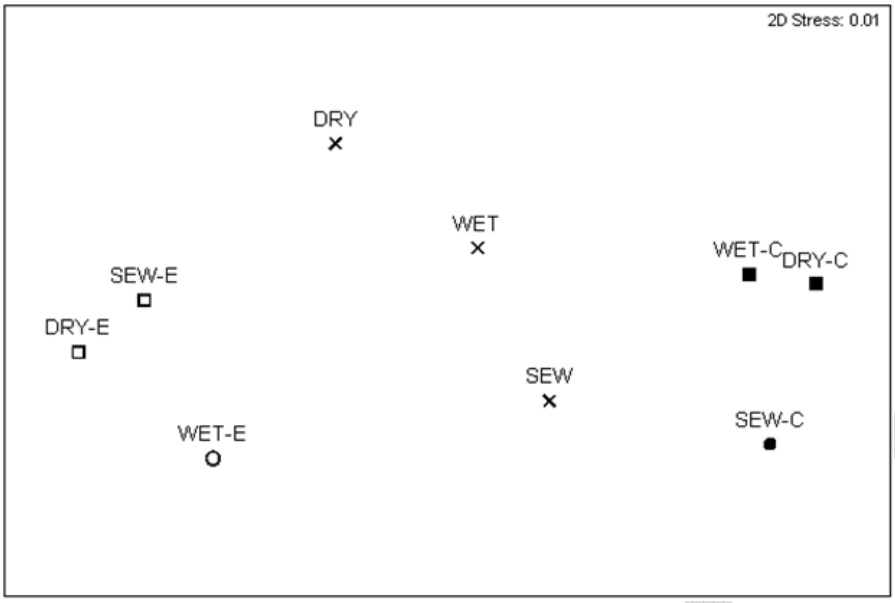


FIGURE 5-2: MDS plot of all source water samples, Colilert and Enterolert enrichments, based on normalized TRFLP peak heights. Grouping based on SIMPROF analysis is indicated by symbols (×, ■, ●, □, ○).

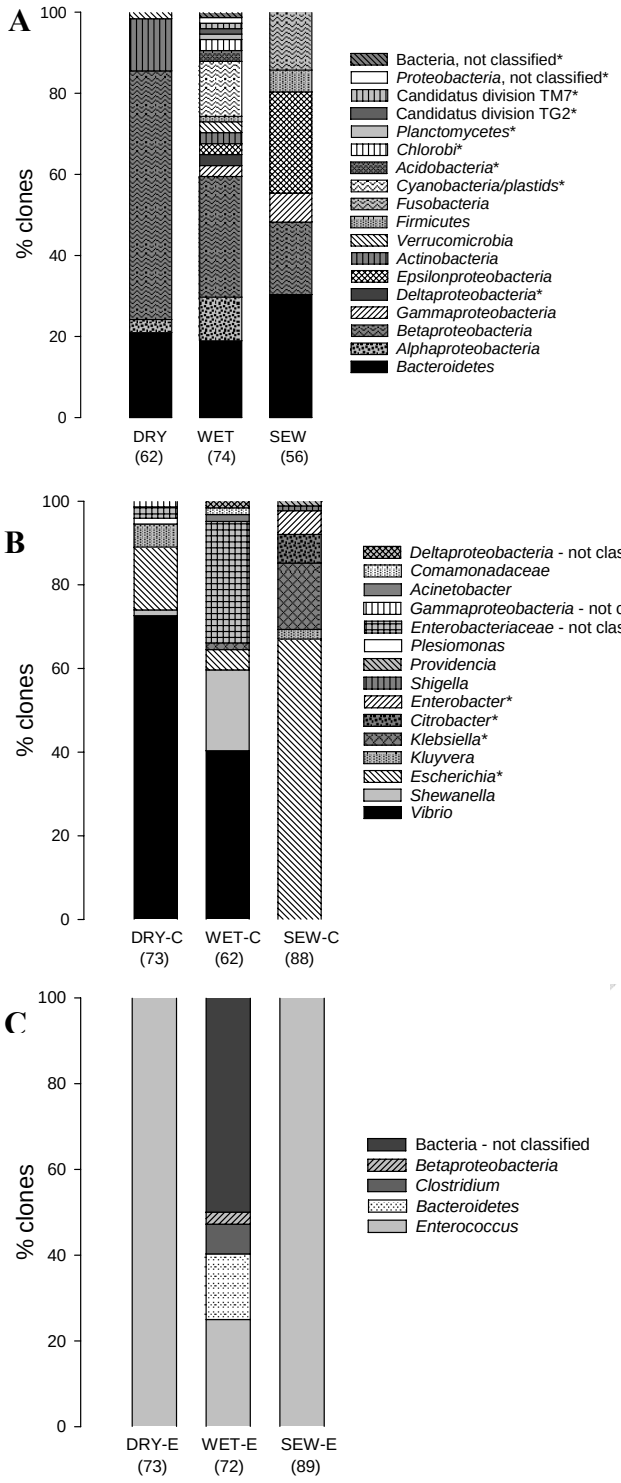
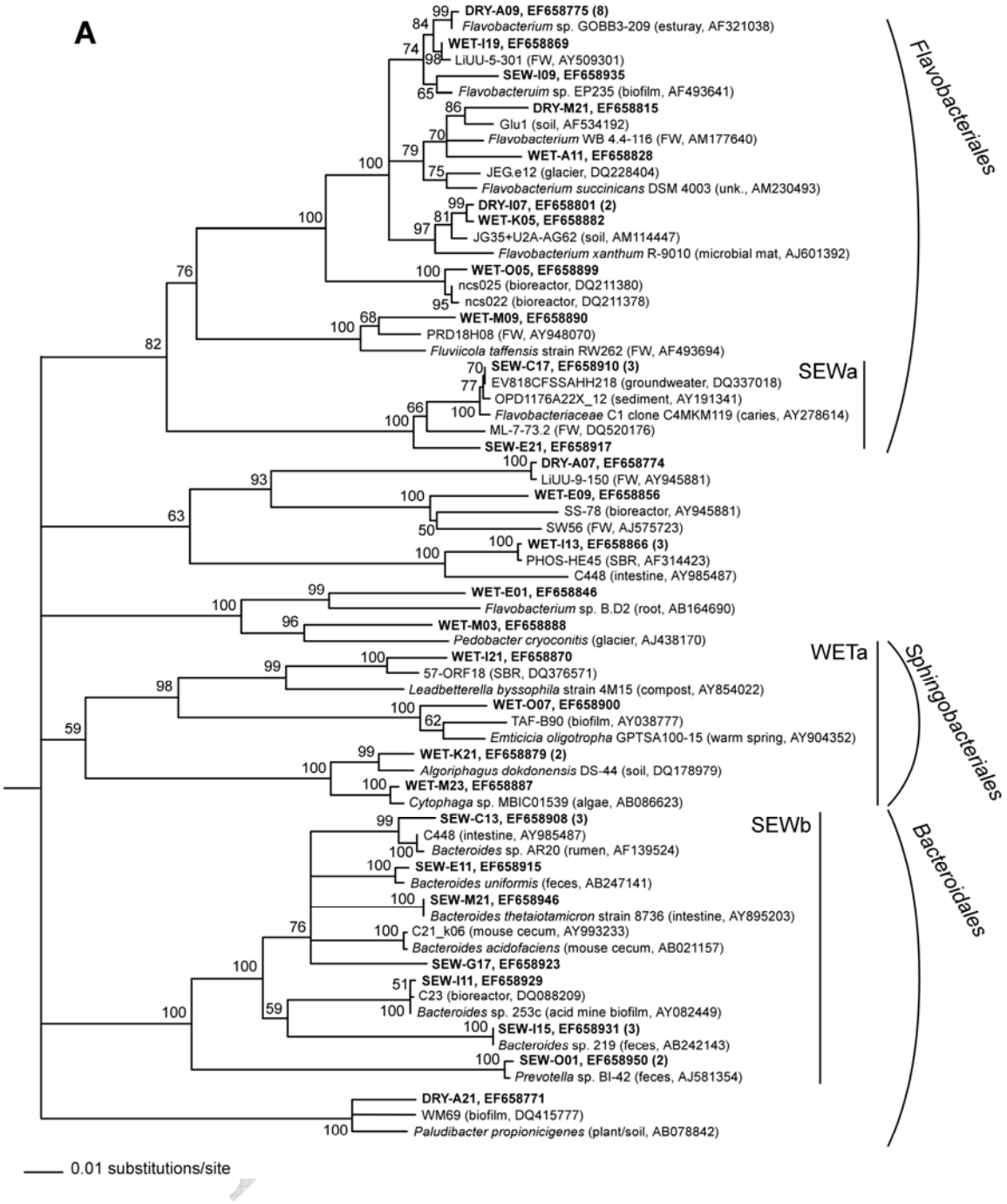


FIGURE 5-3: Composition of the clone libraries for the source water samples (A), pooled yellow/fluorescent wells from Colilert enrichments (B), and pooled fluorescent wells from Enterolert (C) enrichments. The total number of clones is indicated in parentheses. A. Phylogenetic affiliations indicated at phylum/class level. Phylogenetic groups that only occur in sample WET are indicated with *. B. Phylogenetic affiliations indicated at genus level (except clones that could not be classified).

Genera belonging to the *Enterobacteriaceae* are indicated with *. C. Phylogenetic affiliations indicated at phylum/genus level (except clones that could not be classified).


A
DRY-A09, EF658775 (8)
Flavobacterium sp. GOBB3-209 (esturay, AF321038)
WET-I19, EF658869
LiUU-5-301 (FW, AY509301)
SEW-I09, EF658935
Flavobacteruim sp. EP235 (biofilm, AF493641)
DRY-M21, EF658815
Glu1 (soil, AF534192)
Flavobacterium WB 4.4-116 (FW, AM177640)
WET-A11, EF658828
JEG.e12 (glacier, DQ228404)
Flavobacterium succinicans DSM 4003 (unk., AM230493)
DRY-I07, EF658801 (2)
WET-K05, EF658882
JG35+U2A-AG62 (soil, AM114447)
Flavobacterium xanthum R-9010 (microbial mat, AJ601392)
WET-O05, EF658899
ncs025 (bioreactor, DQ211380)
ncs022 (bioreactor, DQ211378)
WET-M09, EF658890
PRD18H08 (FW, AY948070)
Fluviicola taffensis strain RW262 (FW, AF493694)
SEW-C17, EF658910 (3)
EV818CFSSAHH218 (groundweater, DQ337018)
OPD1176A22X_12 (sediment, AY191341)
Flavobacteriaceae C1 clone C4MKM119 (caries, AY278614)
ML-7-73.2 (FW, DQ520176)
SEW-E21, EF658917
SEWa
Flavobacteriales
DRY-A07, EF658774
LiUU-9-150 (FW, AY945881)
WET-E09, EF658856
SS-78 (bioreactor, AY945881)
SW56 (FW, AJ575723)
WET-I13, EF658866 (3)
PHOS-HE45 (SBR, AF314423)
C448 (intestine, AY985487)
WET-E01, EF658846
Flavobacterium sp. B.D2 (root, AB164690)
WET-M03, EF658888
Pedobacter cryoconitis (glacier, AJ438170)
WET-I21, EF658870
57-ORF18 (SBR, DQ376571)
Leadbetterella byssophila strain 4M15 (compost, AY854022)
WET-O07, EF658900
TAF-B90 (biofilm, AY038777)
Emticicia oligotropha GPTSA100-15 (warm spring, AY904352)
WET-K21, EF658879 (2)
Algoriphagus dokdonensis DS-44 (soil, DQ178979)
WET-M23, EF658887
Cytophaga sp. MBIC01539 (algae, AB086623)
WETa
Sphingobacteriales
SEW-C13, EF658908 (3)
C448 (intestine, AY985487)
Bacteroides sp. AR20 (rumen, AF139524)
SEW-E11, EF658915
Bacteroides uniformis (feces, AB247141)
SEW-M21, EF658946
Bacteroides thetaiotamicron strain 8736 (intestine, AY895203)
C21_k06 (mouse cecum, AY993233)
Bacteroides acidofaciens (mouse cecum, AB021157)
SEW-G17, EF658923
SEW-I11, EF658929
C23 (bioreactor, DQ088209)
Bacteroides sp. 253c (acid mine biofilm, AY082449)
SEW-I15, EF658931 (3)
Bacteroides sp. 219 (feces, AB242143)
SEW-O01, EF658950 (2)
Prevotella sp. BI-42 (feces, AJ581354)
SEWb
Bacteroidales
DRY-A21, EF658771
WM69 (biofilm, DQ415777)
Paludibacter propionicigenes (plant/soil, AB078842)
0.01 substitutions/site

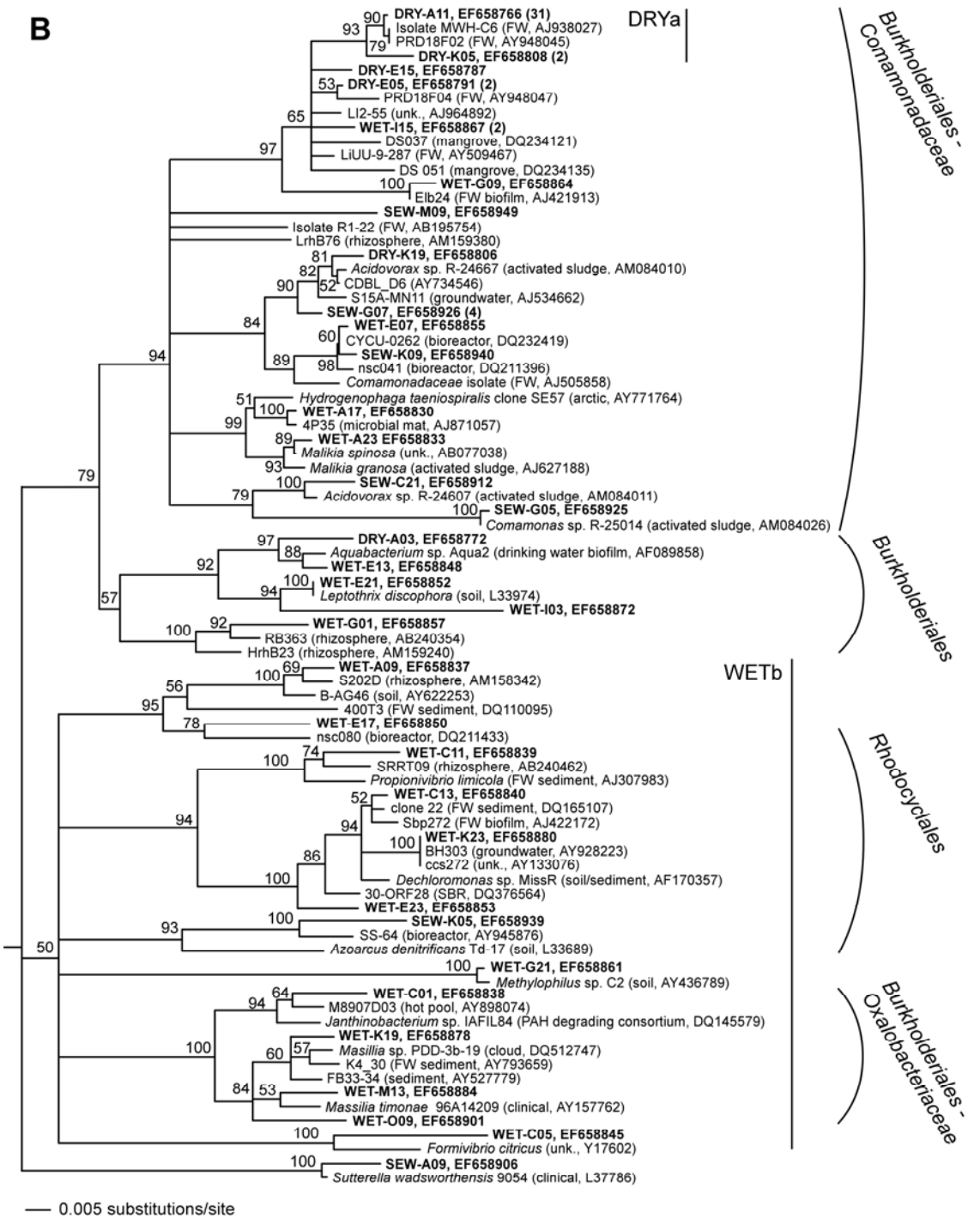


FIGURE 5-4: Phylogenetic relationships among partial 16S rDNA sequences of OTUs from source water samples for (A): *Bacteroidetes*; (B) β-*Proteobacteria*. The percentages of 1000 bootstrap replicates are shown near the relevant nodes in the neighbor-joining trees. Non-supported branches or branches having < 50% bootstrap values were deleted. Clones from this study are boldfaced, and named according to sample name (i.e. DRY, WET, SEW), followed by the clone identification number. GenBank accession numbers are indicated following the comma. Reference bacteria are indicated by clone or isolate name, followed by the isolation source and accession numbers between brackets. *Nitrospira marina* was used as outgroup for all trees (not shown). The number of clones per OTU, if more than 1, is indicated in parentheses.

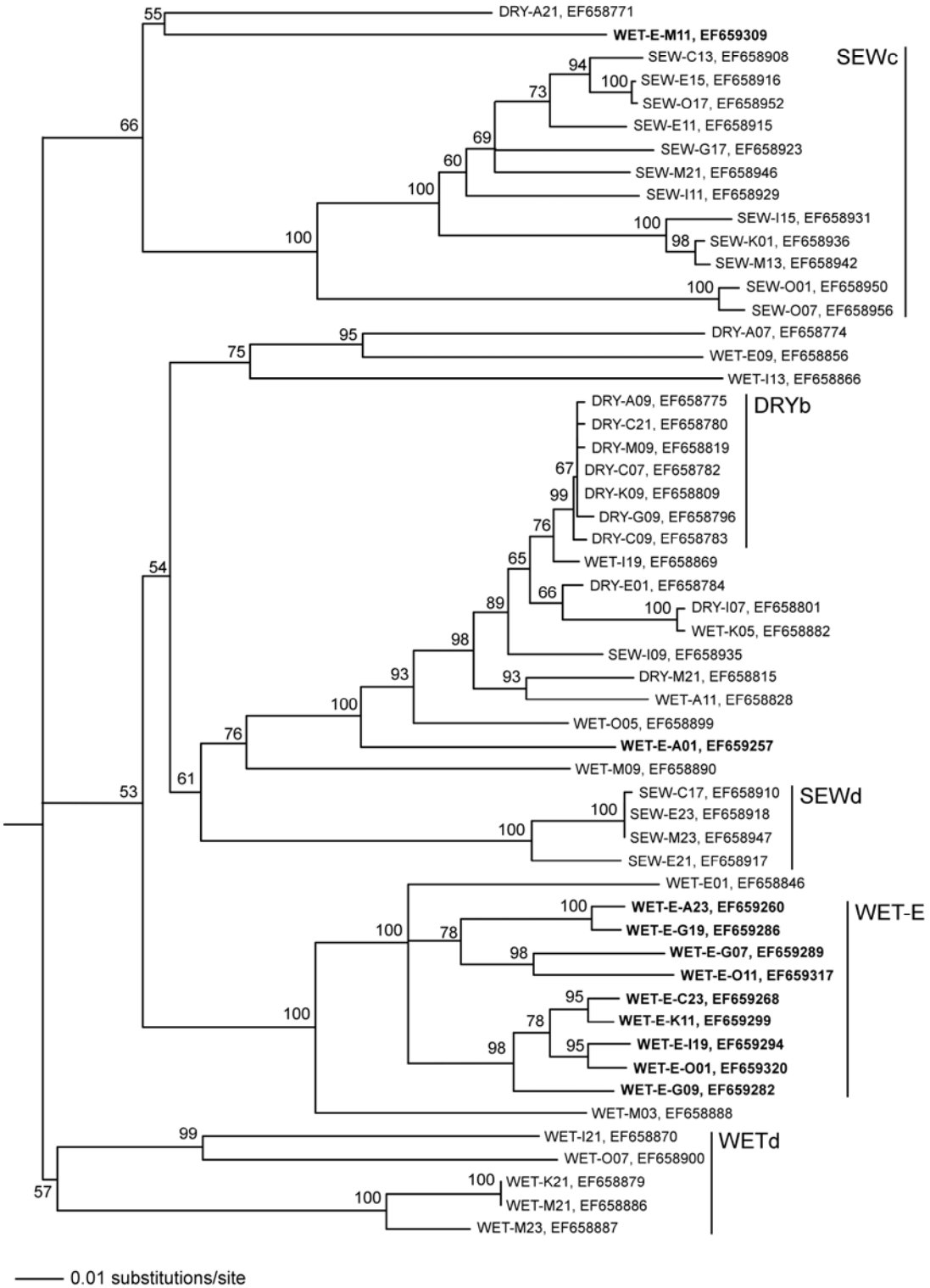


Figure 5-5: Phylogenetic relationships among all *Bacteroidetes* partial 16S rDNA sequences. The percentages of 1000 bootstrap replicates are shown to the left of the relevant nodes in the neighbor-joining trees. Non-supported branches or branches having < 50% bootstrap values were deleted. Clones are named according to sample location (i.e. DRY, WET, SEW), with suffix "E" for the clones from the Enterolert enrichment (boldfaced), followed by clone number. GenBank accession numbers are indicated following the comma. *Nitrospira marina* was used as outgroup (not shown)

Chapter 6: High Density Microarray Analysis of Water Quality in a California Coastal Creek (Phase III PhyloChip analysis)

6.1 Introduction

One implication of human waste discharges into surface waters is that bacterial human pathogens are also discharged. Even knowing what pathogen groups are present can help put into context the possible risk to human health of discharges as described in Chapter 2. In this phase of research, a new technology was employed to further interrogate DNA extracted from Phase II Mission Creek "snapshot" samples. The objective was to determine if pathogen groups could be discerned from these samples.

Microarray technology is particularly useful in assessing microbial diversity. A high-density microarray (PhyloChip) has been developed by the laboratory of Gary Andersen at Lawrence Berkeley National Laboratory to comprehensively profile microbial communities. This high-density microarray is able to examine the known diversity of prokaryotes in environmental samples. To date, the PhyloChip has been applied towards characterization of metal-reducing bacteria during uranium bioremediation (Brodie, DeSantis et al. 2006) and flux in airborne prokaryote populations in urban settings (Brodie, DeSantis et al. 2007). The two key features that are the hallmark of the PhyloChip are i) multiple probes that in unison identify the presence of a specific prokaryotic species, and ii) a physically adjacent mismatch control probe to minimize the effect of non-specific hybridization. To maximize the numbers of species that the PhyloChip can identify, hundreds of thousands of oligonucleotide probes for each array are required. Results across divergent environmental samples have demonstrated high correlation with split samples used for clone library sequence analysis. Although a very small number of completely novel bacterial sequences not seen on the PhyloChip have been observed in the clone libraries, the array data has identified additional, low-abundance sequences not observed in clone libraries. While this microarray is unreliable in classifying novel taxa it is capable of confirming the majority of clone-detected sub-families in addition to revealing greater richness, even at the phylum level.

Furthermore, array-observed richness corresponded well with non-parametric richness predictions calculated from clone sampling indicating a more complete inventory of the ecosystems sampled (DeSantis, Brodie et al. 2007). The laborious, costly and time-consuming nature of clone library analysis diminishes its utility in studies requiring replication and temporal monitoring. The responsiveness of the 16S rDNA microarray to nucleic acids from diverse phyla in complex mixtures and its suitability for investigations requiring replication, demonstrates a necessary advance toward the goal of high-throughput ecological monitoring. For these reasons, we believe the high-density DNA microarray offers a promising approach for studies of microbial ecology, and more specifically for microbial water quality research or monitoring.

6.2 Materials and Methods

6.2.1 Microarray Design

In summary, a collection of 148,925 16S rDNA probes, comprising just under 9000 OTUs (Operational Taxonomic Units), were spotted onto glass slides. An OTU may consist of one to hundreds of bacterial species and can have more than one genus in it. Environmental DNA extracts were PCR amplified, fragmented, biotinylated, and hybridized to the microarray chip. The microarray probe design approach previously described for differentiating Staphylococcacae (DeSantis, Dubosarskiy et al. 2003) was applied to the all known prokaryotic sequences of substantial length. Briefly, 16S rDNA sequences (*Escherichia coli* base pair positions 47 to 1473) were obtained from approximately 30,000 16S rDNA sequences that were at least 600 nucleotides in length in the 15 March 2002 release of the prokMSA database 16S rDNA database, greengenes (www.greengenes.lbl.gov). This region was selected because it is bounded on both ends by universally conserved segments that can be used as PCR priming sites to amplify bacterial or archaeal (Dojka, Hugenholtz et al. 1998) genomic material using only 2 to 4 primers. Putative chimeric sequences were filtered from the data set using the software package Bellerophon (Huber, Faulkner et al. 2004) preventing them from being misconstrued as novel organisms (Hugenholtz and Huber 2003). The filtered sequences

are considered to be the set of putative 16S rDNA amplicons. Sequences were clustered to enable each sequence of a cluster to be complementary to a set of perfectly matching (PM) probes. Putative amplicons were placed in the same cluster as a result of common 17-mers found in the sequence. The resulting 8,988 clusters, each containing less than 5% sequence diversity, were considered OTUs representing all 262 known prokaryotic orders. The taxonomic family of each OTU was assigned according to the placement of its member organisms in Bergey's Taxonomic Outline. The Hugenholtz Taxonomic Outline was consulted for phylogenetic classes containing uncultured environmental organisms or unclassified families belonging to named higher taxa. The OTUs comprising each family were clustered into subfamilies, each containing 85% transitive sequence identity according to a previously described method (DeSantis, Dubosarskiy et al. 2003). Altogether, 842 subfamilies were found. The taxonomic position of each OTU as well as the accompanying NCBI accession numbers of the sequences composing each OTU can be viewed at http://greengenes.lbl.gov/Download/Taxonomic_Outlines/G2_SeqDescByOTU_outline.txt.

The objective of the probe selection strategy was to obtain an effective set of probes capable of correctly categorizing mixed amplicons into their proper OTU. For each OTU, a set of 11 or more specific 25-mers (probes) were sought that were prevalent in members of a given OTU but were dissimilar from sequences outside the given OTU were sought. In the first step of probe selection for a particular OTU, each of the sequences in the OTU were separated into overlapping 25-mers, the potential targets. Then each potential target was matched to as many sequences of the OTU as possible. Since each sequence in an OTU could range from 600 to 1,500 nucleotides, it was not sufficient to use a simple text search. Therefore, the multiple sequence alignment provided by greengenes was necessary to provided discrete measurement of group size at each potential probe site. For example, if an OTU containing seven sequences possessed a probe site where one member was missing data, then the site-specific OTU size was only six. In ranking the possible targets, those having data for all members of that OTU were preferred over those found only in a fraction of the OTU members. In the second step, a subset of the prevalent targets were selected and complemented into probe

orientation, avoiding those capable of mis-hybridization to an unintended amplicon. Probes presumed to have the capacity to mis-hybridize were those 25-mers that contained a central 17-mer matching sequences in more than one OTU (Urakawa, El Fantroussi et al. 2003). Thus, probes that were unique to an OTU solely due to a distinctive base in one of the outer four bases were avoided. Also, probes with mis-hybridization potential to sequences having a common tree node near the root were favored over those with a common node near the terminal branch. As each PM probe was chosen, it was paired with a control 25-mer (mismatching probe, MM), identical in all positions except the thirteenth base. The MM probe did not contain a central 17-mer complimentary to sequences in any OTU. The target probe and MM probes constitute a probe pair analyzed together. The chosen oligonucleotides were synthesized by a photolithographic method at Affymetrix Inc. (Santa Clara, CA, USA) directly onto a 1.28 cm by 1.28 cm glass surface at an approximate density of 10,000 probes per μm^2 (Chee, Yang et al. 1996). Each unique probe sequence on the array had a copy number of roughly 3.2×10^6 (personal communication, Affymetrix). The entire array of 506,944 features was arranged as a square grid of 712 rows and columns. Of these features, 297,851 were oligonucleotide 16S rDNA PM or MM probes, and the remaining were used for image orientation, normalization controls, or other unrelated analyses. Each DNA chip has two kinds of controls on it: i) probes that target amplicons of prokaryotic metabolic genes spiked into the 16S rDNA amplicon mix in defined quantities just prior to fragmentation and ii) probes complimentary to a pre-labeled oligonucleotide added into the hybridization mix. The first control collectively tests the fragmentation, biotinylation, hybridization, staining and scanning efficiency. It also allows the overall florescent intensity to be normalized across all the arrays in an experiment. The second control directly assays the hybridization, staining and scanning. An additional control can be added by testing the PCR negative control product. Any OTUs present in this control can be subtracted from the other samples' results prior to further analysis. It is possible through this to detect small amounts of amplicon that are not visible by gel analysis only.

6.2.2 16S rDNA Amplification

The 16S rDNA was amplified from the gDNA using non-degenerate Bacterial primers 27F.jgi and 1492.jgi (GGT TAC CTT GTT ACG ACT T) (almost always) and the universal Bacterial primers 27f.1 (5' AGR GTT TGA TCM TGG CTC AG) and 1492R (5' GGT TAC CTT GTT ACG ACT T) (when DNA template was limiting (this occurred for one amplification of samples from sets 5 – 8)). Polymerase chain reaction (PCR) was carried out using the *TaKaRa Ex Taq* system (Takara Bio Inc, Japan). Each PCR reaction mix contained 1X *Ex Taq* buffer, 200 uM total final concentration of *TaKaRa* dNTP mixture, 0.02U/μL *TaKaRa Ex Taq* polymerase, 0.4mg/mL bovine serum albumin (BSA), and 300 pmol of each primer. PCR conditions were 1 cycle of 3 min at 95°C, followed by 35 cycles of 30 sec at 95°C, 30 sec at 48-58°C (gradient block), and 1 min at 72°C, and finishing with 7 min incubation at 72°C. The gradient PCR was used to optimize the microbial diversity detection. This part of the technique was designed by the Joint Genome Institute (Walnut Creek, CA) to maximize microbial diversity amplification from environmental samples. When the universal primers were used, only two temperatures were used (50 and 56 degrees C) to minimize extraneous product formation.

6.2.3 Microarray Processing

For each array, amplicons were concentrated to a volume less than 40 μl by isopropanol precipitation. The PCR products (500 ng) were spiked with known concentrations of amplicons derived from prokaryotic metabolic genes. This mix was fragmented to 50-200 bp using DNAse I (0.02 U/μg DNA, Invitrogen) and One-Phor All buffer per Affymetrix's protocol. The complete mixture was incubated at 25 °C for 10 min., 98°C for 10 min., and then labeled. Biotin labeling was accomplished using the GeneChip Labeling Reagent (Affymetrix) per the manufacturer's directions. The labeled DNA was then denatured (99 °C for 5 min) and hybridized to the DNA microarray at 48 °C overnight (> 16 hr). The arrays were subsequently washed and stained. Reagents, conditions, and equipment are detailed elsewhere (Masuda and Church 2002).

6.2.4 Scanning and Probe Set Scoring

Arrays were scanned using a GeneArray Scanner (Affymetrix, Santa Clara, CA, USA). The scan was recorded as a pixel image and analyzed using standard Affymetrix software (Microarray Analysis Suite, version 5.1) that reduced the data to an individual signal value for each probe. Background probes were identified as those producing intensities in the lowest 2% of all intensities. The average intensity of the background probes was subtracted from the fluorescence intensity of all probes. The noise value (*N*) was the variation in pixel intensity signals observed by the scanner as it read the array surface. The standard deviation of the pixel intensities within each of the identified background cells was divided by the square root of the number of pixels comprising that cell. The average of the resulting quotients was used for *N* in the calculations described below.

Probe pairs scored as positive were those that met two criteria: i) the intensity of fluorescence from the perfectly matched probe (PM) was greater than 1.3 times the intensity from the mismatched control (MM), and ii) the difference in intensity, PM minus MM, was at least 130 times greater than the squared noise value (>130 N^2). The positive fraction (PosFrac) was calculated for each probe set as the number of positive probe pairs divided by the total number of probe pairs in a probe set. A subfamily was considered present when at least one of its subordinate OTUs had a PosFrac > 0.92 in all three replicates.

6.2.5 Data Analysis

The CEL files obtained from the Affymetrix software that produces information about the fluorescence intensity of each probe (PM, MM, and control probes) were analyzed using the CELanalysis software designed by Todd DeSantis (LBNL, Berkeley, USA). The output is further processed by combining the blank samples from set 5 and 6 to determine which OTUs to remove from further analysis. If an OTU was present in both blanks, it was removed. Any OTU in the PCR negative control also was removed. To be

present, the OTU had to have at least 90% of the probe pairs in the set be positive (see above criteria). This is the pf cutoff of 0.9. For the remaining OTUs, any OTU with a fluorescence intensity at least 25% greater than the average of the two blanks' fluorescence intensity was also considered present in any given sample. For each paired flight set (e.g., 5N and 5RN (the return flight)), the OTUs present in both flights were considered 'shared.'

6.2.6 Samples Analyzed

The Phylochip was used to analyze samples from the dry weather snapshot study (Mission Creek, 6/28/05 – 6/30/05, see Chapter 2), and additional sewage and human fecal material reference samples. Table 6-1 gives an overview of the sampling IDs, times and location. Sampling and DNA extraction was performed as described before. The extracted DNA was PCR amplified and analyzed using the PhyloChip as described above.

6.3 Results

6.3.1 Microbial Community Composition using PhyloChip

The PhyloChip detected 2299 OTUs in total for all samples. The truncated dataset still included 909 OTUs. Truncation consisted of only including OTUs that had a PosFrac > 0.92 in at least one of the samples, and of only including 1 representative OTU per subfamily. Fig. 6-1 shows the general clustering of all samples, based on the truncated dataset. The clustering to the left shows the phylogenetic clustering, while the top clustering the clustering of the samples. The latter showed that samples 1, 3, 6 and 9 clustered well within each site (all 3 dates clustered together), and samples 5 and 7 also formed one cluster, except for S05. The human fecal sample was most distantly related from all other samples, while all Westside Drain samples (9) clustered most closely to the sewage samples. Other samples included in the large cluster with the sewage, but more distantly related were: all Haley Drain (6), all OMC into MC (8), all Laguna Channel (3), two Laguna Lagoon samples (2), and one Montecito sample. The range of human-

specific *Bacteroides* concentrations for each sample was indicated using * or ** symbols, and was not related with the clustering of the samples.

The clustering of samples was also performed using a subset of OTUs that were only related to fecal indicator bacteria (FIB), such as total coliforms (only *Enterobacteriaceae*), enterococci (only *Enterococcaceae*) and *Bacteroidaceae*/*Prevotellaceae*. When only including *Enterobacteriaceae* subfamilies (Fig. 6-2), a very different clustering was observed. First of all, the samples related with human fecal waste (S1024, sewage and human) did not cluster together. However, most 1, 4, 5 and 7 samples clustered separate from the cluster containing the human fecal material samples, as was observed when including all subfamilies. No dominant clustering according to location was observed anymore. A lot of OTUs belonging to the *Enterobacteriaceae* were present in all samples. When including only *Enterococcaceae* subfamilies (Fig. 6-3), the sewage samples clustered very closely together with the S21 sample (ocean). Also, the samples did not cluster according to location anymore. Similar as with the *Enterobacteriaceae*, most OTUs belonging to the *Enterococcaceae* were present in all samples. Including only *Bacteroidaceae*/*Prevotellaceae* subfamilies (Fig. 6-4) clustered the human fecal waste samples separate. The water samples were divided into two groups, one containing all samples 5, 6, 7 and S24 and S28, and one containing all remaining samples. For *Bacteroidaceae*/*Prevotellaceae*, some OTUs were abundant in all samples (e.g. 5256, 5320,…), some had a general low abundance (e.g. 5966, 6216), while some were especially abundant in the human fecal waste samples (e.g. most OTUs in the upper half of Fig. 6-4).

Similar as with the analysis of all OTUs using the truncated dataset, no clustering according to human-specific *Bacteroides* concentrations was observed with any of the above OTU data subsets.

6.3.2 Occurrence of FIB using PhyloChip

In order to compare FIB abundance between the IDEXX assays and the PhyloChip, we averaged the hybridization intensities of all OTUs indicating the presence of *Enterococcus* spp. per site, and compared this signal with the culture-based FIB counts

(Fig. 6-5, Fig. 6-6). The hybridization intensities of all OTUs were rather constant, while the culture-based IDEXX *Enterococcus* spp. counts varied orders of magnitude between times and sites. The correlation plots indicated no relation between the IDEXX and PhyloChip abundances.

A similar analysis was not performed for total coliforms, because 9 out of 27 samples were out of range for the Quantitray/2000 quantification. No OTUs containing *E. coli* were detected in the truncated dataset.

6.3.3 Occurrence of OTUs Representing Human Pathogens

Based on a literature search (Ford 1999; Straub and Chandler 2003; Cangelosi, Freitag et al. 2004), we compiled a list of pathogenic genera/species of environmental importance (Table 6-2). Consequently, the PhyloChip results were scanned for the occurrence of OTUs that contained those pathogenic genera/species. The raw dataset was used for this analysis, in order to obtain a maximum sensitivity. Based on the absence of OTUs containing the pathogens *Bacillus anthracis*, *Borrelia burgorferi*, *Rickettsia typhi*, *Listeria monocytogenes, Coxsiella burnetti* and *Campylobacter jejuni*, we could rule out the presence of these pathogens in our samples. However, based on the presence of the OTUs listed in Table 6-2, we could not rule out the presence of other pathogens. In some cases, the presence of a pathogenic species could be confirmed (*Helicobacter pylori*, *Vibrio cholerae*, some *Salmonella* species), although in most cases, OTUs indicated the presence of both non-pathogenic and pathogenic species from the same or a different genus.

In Fig. 6-7, an overview is shown of the hybridization intensities of all pathogen-related OTUs across all samples. Again, no clustering according to the human-specific *Bacteroides* concentrations is observed. Two major clusters were formed. One cluster included all samples 1 and 7, and two of the 4 and 5 samples. The other major cluster included the human fecal waste samples and all remaining samples. The samples did not cluster well according to sampling location. In general, the OTUs associated with *Mycobacterium avium* and *Vibrio cholerae* produced the lowest signal. The other OTUs produced a rather similar signal across all samples. The OTUs associated with

Aeromonas spp. and *Helicobacter pylori* appeared most abundant in general. Moreover, S26 showed the highest intensities for *Aeromonas* spp., *Helicobacter pylori* and *Salmonella* spp. Finally, none of the OTUs associated with pathogens could be related with the human-specific *Bacteroides* marker.

6.4 Discussion

As the PhyloChip is a very recent technology, and has never been used in the field of microbial water quality, we determined in what way we could compare the PhyloChip results with other techniques, currently in use.

First, we found that no relation could be found between between Quantitray/2000 *Enterococcus* spp. concentrations and the relative abundance of all OTUs related with *Enterococcus* spp. in the PhyloChip. We could only assess if the relative *Enterococcus* abundances across samples varied in a similar way, as the PhyloChip does not allow determining absolute concentrations. The data did not lend themselves to perform a similar assessment for total coliforms of *E. coli*. The disagreement between both methods could be due to the discrepancy between culture-based and DNA-based methods, because non-culturable cells are detected using the PhyloChip. Previous reports have indicated that the PhyloChip results correlated well with quantitative PCR for selected targets, so the quantitativeness of the PhyloChip should not be a limiting factor.

Second, we found that the clustering of samples based on the PhyloChip results, did not agree with grouping according to human-specific *Bacteroides* concentrations. However, analyzing the clustering more in detail indicated that a general pattern of clustering could be distinguished. All clustering, based on all possible subfamilies, or only *Enterobacteriaceae*, *Enterococcaceae* or pathogen-associated OTUs, indicate that the human fecal waste samples cluster with 6 out of 8 samples containing human-specific *Bacteroides*, but also with all samples 8 (OMC into MC) and 9 (Westside Drain). The clustering disagreed with the human-specific *Bacteroides* data in that samples S25 and S27 were never included in the major cluster containing the human fecal waste samples. In other words, the samples 8 and 9 contain a microbial community more similar to those of samples containing human fecal waste, or the human-specific *Bacteroides* marker, than to the creek water samples between Haley Creek and the ocean. More research

should determine if this is really due to the presence of human fecal waste, or if it is caused by a dominant effect of the water microbial community, not related to human fecal waste. One way to approach this further research, could be to design a similar microarray, but containing probe sets for known human specific markers.

The cluster analysis based on the *Bacteroidaceae*/*Prevotellaceae* was performed because the human-specific *Bacteroides* marker is located in the *Bacteroides*/*Prevotalla* cluster (Bernhard and Field 2000). This clustering was very different than the clustering based on all other families. The human fecal waste samples did not cluster to any of the other water samples, and the latter did not cluster in groups as before. A group of phylogenetically clustered OTUs were highly abundant in the human fecal waste samples, but not in any of the other water samples. These OTUs could be candidate-markers for human fecal waste.

We compiled a list of pathogens of interest, and determined what OTUs were indicative for the presence of these pathogens. Using this approach, we could rule out the presence of a number of pathogens in our samples. Also, we could show that the pathogenic taxa *Aeromonas* spp., *Helicobacter pylori*, *Leptospira* spp., *Salmonella* spp. and *Vibrio* cholerae were present in all water samples, with the first two taxa being most abundant. For the other pathogenic taxa, the signal was confounded by the simultaneous detection of pathogenic and non-pathogenic species in the same OTU. Especially for these taxa, other probes should be included in the microarray, to ensure a specific signal for pathogens. Moreover, non 16S rDNA probes should be included, as proper identification of pathogenic serotypes require the analysis of other genes, e.g. O-serotype-specific genes (Li, Liu et al. 2006).

6.5 Conclusions

PhyloChip analysis could distinguish microbial communities of freshwater based on their relatedness with human fecal waste (fecal samples and sewage). The majority of samples containing the human-specific *Bacteroides* marker were grouped with human fecal waste samples, although the agreement between both methods was not complete. Potential new markers for human fecal waste were identified (a cluster within the *Bacteroidaceae*/*Prevotellaceae* group). Finally, the absence of important water-related

pathogens (e.g. *Campylobacter jejuni*), and the presence of other pathogens (e.g. *Helicobacter pylori*) was shown. The results obtained in this study will guide the further development of a microarray designed specifically for microbial water quality purposes.

6.6 Acknowledgements

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TABLE 6-1: Overview of the PhyloChip sampling IDs and sample locations. All Mission Creek watershed samples (1 – 9) were taken at 3 dates: 6/28/05 (date 0), 6/29/05 (date 1) and 6/30/05 (date 2). Sample assignments were S, followed by date, followed by sample ID (e.g. S24).

Location	Phylochip ID
Ocean surf	1
Laguna lagoon	2
Laguna channel	3
Mission lagoon	4
Montecito	5
Haley-drain	6
Haley-creek	7
OMC into MC	8
Westside drain	9
El Estero WWTP (12/14/04)	sewage
El Estero WWTP (10/24/05)	S1024
Human fecal (12/15/04)	human

Table 6-2: Pathogen genera/species of potential importance in water samples, grouped into water-associated pathogens or general environmental pathogens. The OTU description indicates what species hybridize with the OTU number.

Pathogen genus/species	OTU	OTU description
Water		
Aeromonas spp.	8340, 8364, 8621, 9000, 9026, 9062, 9440, 9494	*Aeromonas* spp.
Campylobacter jejuni	n.d.	
Helicobacter pylori	10442	*H. pylori* + *Helicobacter* spp.
	10443	*H. pylori* (10) + *H. nemestrinae* (1)
	10518	*H. nemestrinae*
	10534	*H. pylori*
Legionella pneumophila	8220, 8836	*L. pneumophila* + *Legionella* spp.
Leptospira spp.	6496	*Leptospira* spp.
Mycobacterium avium	1650	*M. avium* + *Mycobacterium* spp.
Salmonella spp.	8430	*S. bongori*
	8640	*Salmonella* spp.
	8886	*S.* subsp *enterica*, S. *typhimurum*, *S.* serovar *typhi*, *S. typhi*
	8974	*S.* subsp *enterica*
	9358	(see *Shigella* spp.)
Shigella spp.	9358	*E. coli, Shigella flexneri, Shigella dysenteriae, Shigella boydii, Hafnia alvei, Citrobacter koseri, Klebsiella planticola, Salmonella* spp.
Vibrio cholerae	9225	*Vibrio cholerae*
Vibrio parahaemolyticus	8665	*Vibrio parahaemolyticus* + *Vibrio* spp.
Yersinia enterocolytica	9262	*Y. enterocolytica* + *Yersinia* spp.
General environmental		
Bacillus anthracis	n.d.	
Bartonella henslei	7634	*Bartonella henslei* + *Bartonella* spp.
Borrelia burgorferi	n.d.	
Burkholderia cepacia	7837, 8044, 8097	*B. cepacia* + *Burkholderia* spp.
Coxsiella burnetti	n.d.	
Listeria monocytogenes	n.d.	
Rickettsia typhi	n.d.	

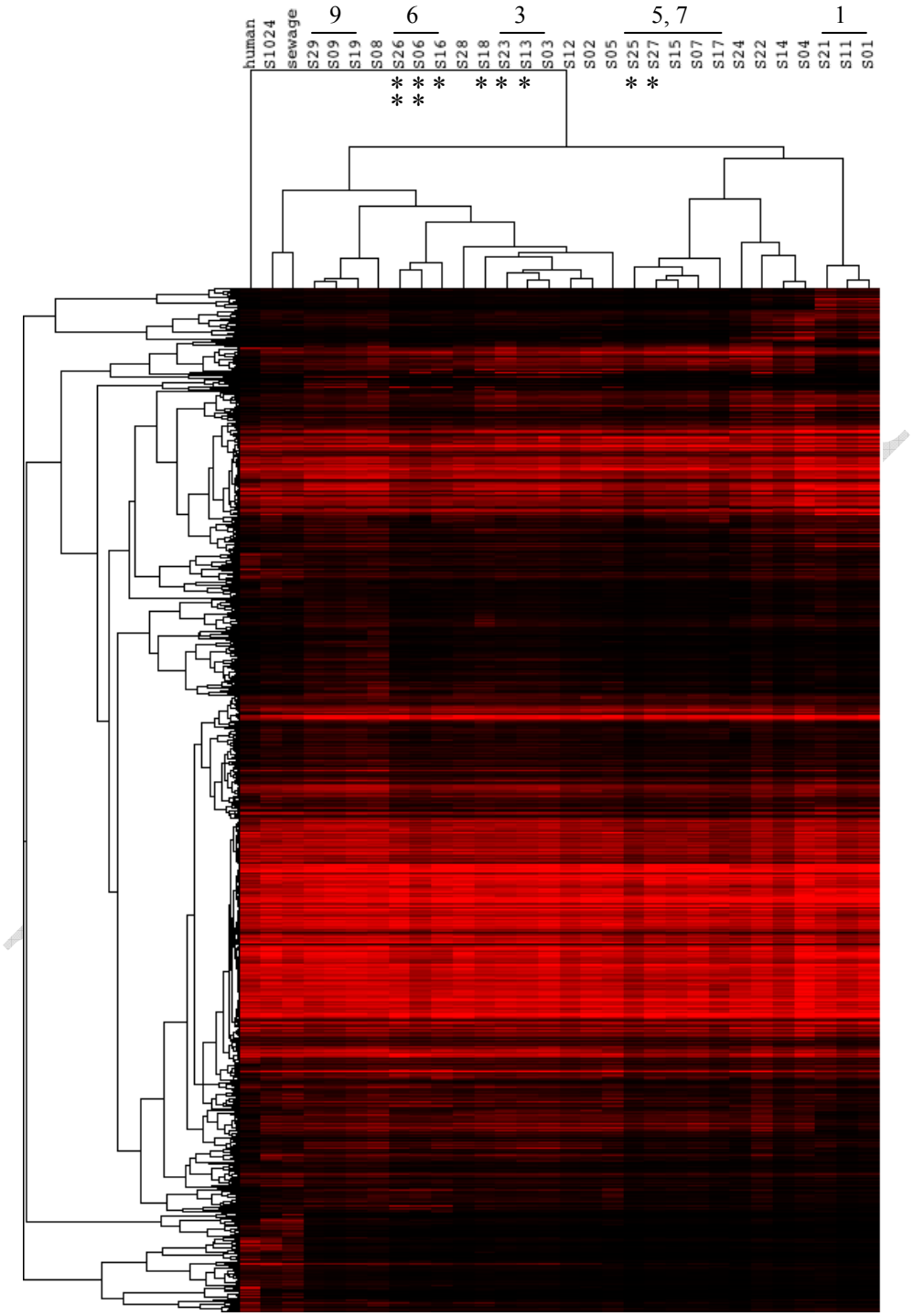

FIGURE 6-1: Hierarchical cluster analysis for all samples based on the truncated dataset. Clustering on the left shows the phylogenetic clusters, top clustering shows the sample clusters. Red intensities indicate the magnitude of the hybridization signal. The range of the human-specific *Bacteroides* concentrations are indicated for all samples (except human, S1024, sewage): * = 3.8 x 10^3 – 1.7 x 10^5 markers l^{-1}, ** = 4.2 x 10^6 – 1.5 x 10^7 markers l^{-1}, no mark = below detection limit.

FIGURE 6-2: Hierarchical cluster analysis of all samples based on *Enterobacteriaceae* OTUs, using the raw dataset. The top clustering shows the sample clusters, the numbers on the right indicate the OTUs. Red intensities indicate the magnitude of the hybridization signal. The range of the human-specific *Bacteroides* concentrations are indicated for all samples (except human, S1024, sewage): * = 3.8 x 10^3 – 1.7 x 10^5 markers l^{-1}, ** = 4.2 x 10^6 – 1.5 x 10^7 markers l^{-1}, no mark = below detection limit.

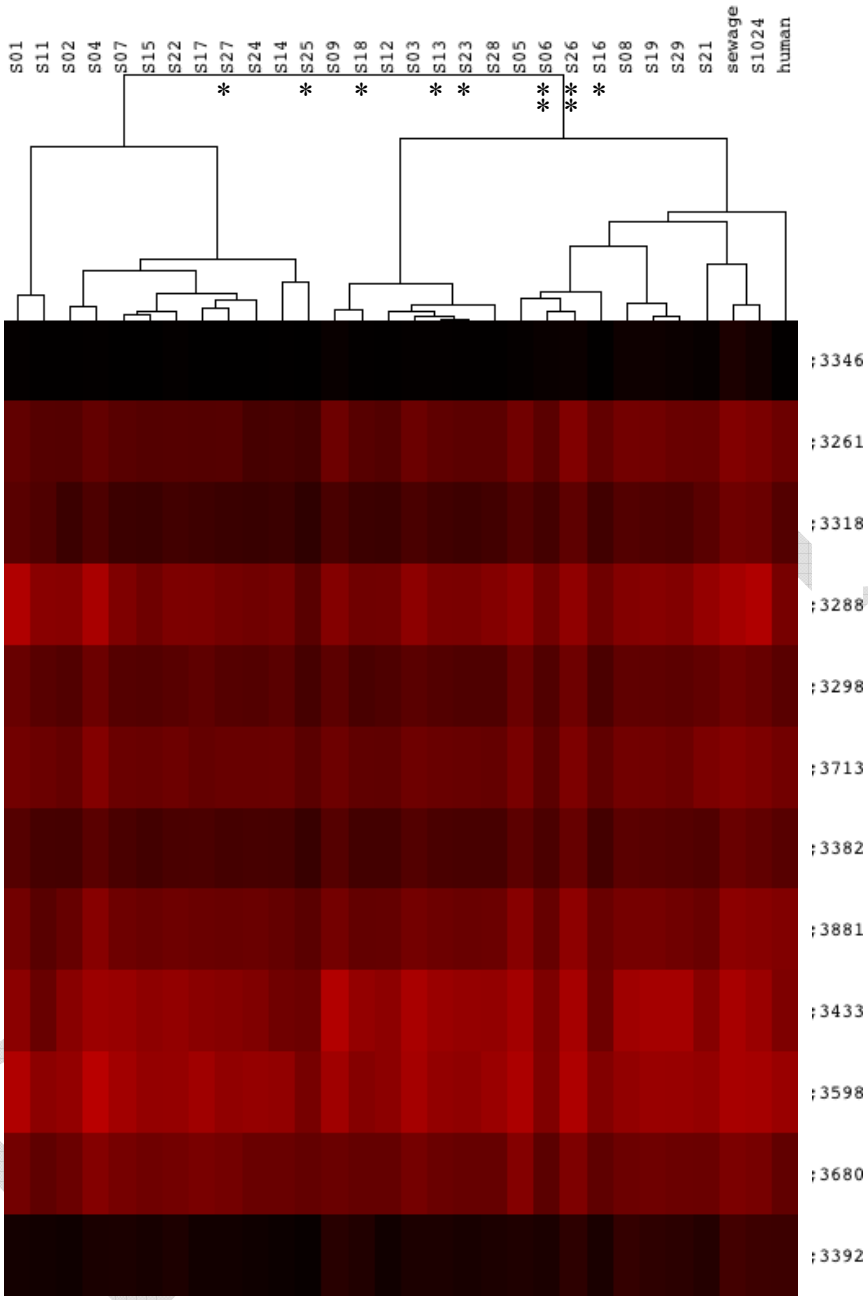

FIGURE 6-3: Hierarchical cluster analysis of all samples based on *Enterococcaceae* OTUs, using the raw dataset. The top clustering shows the sample clusters, the numbers on the right indicate the OTUs. Red intensities indicate the magnitude of the hybridization signal. The range of the human-specific *Bacteroides* concentrations are indicated for all samples (except human, S1024, sewage): * = $3.8 \times 10^3 - 1.7 \times 10^5$ markers l^{-1}, ** = $4.2 \times 10^6 - 1.5 \times 10^7$ markers l^{-1}, no mark = below detection limit.

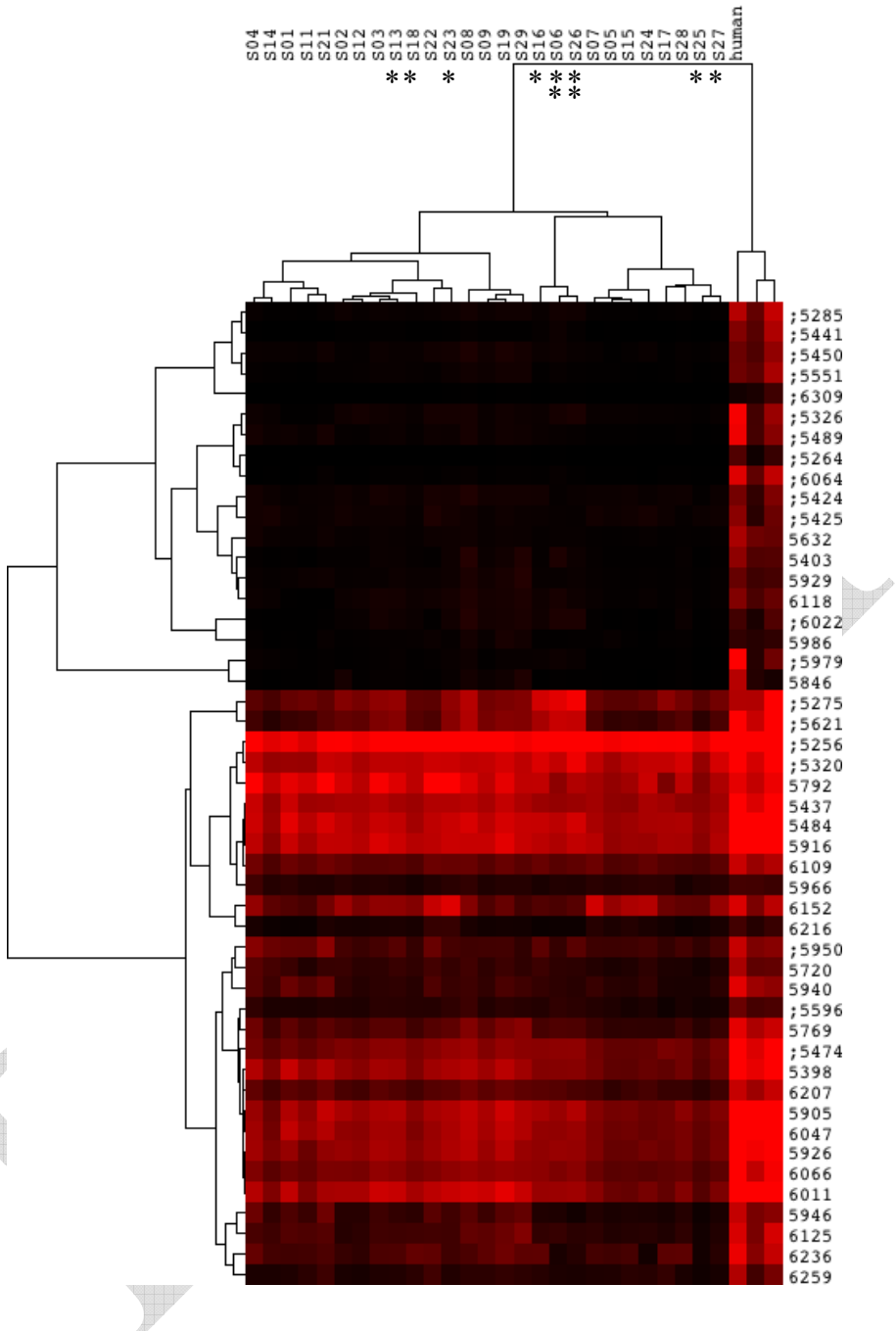

FIGURE 6-4: Hierarchical cluster analysis of all samples based on *Bacteroidaceae - Prevotellaceae* OTUs, using the raw dataset. The top clustering shows the sample clusters, the numbers on the right indicate the OTUs. Red intensities indicate the magnitude of the hybridization signal. The range of the human-specific *Bacteroides* concentrations are indicated for all samples (except human, S1024, sewage): * = 3.8 x 10^3 – 1.7 x 10^5 markers l^{-1}, ** = 4.2 x 10^6 – 1.5 x 10^7 markers l^{-1}, no mark = below detection limit.

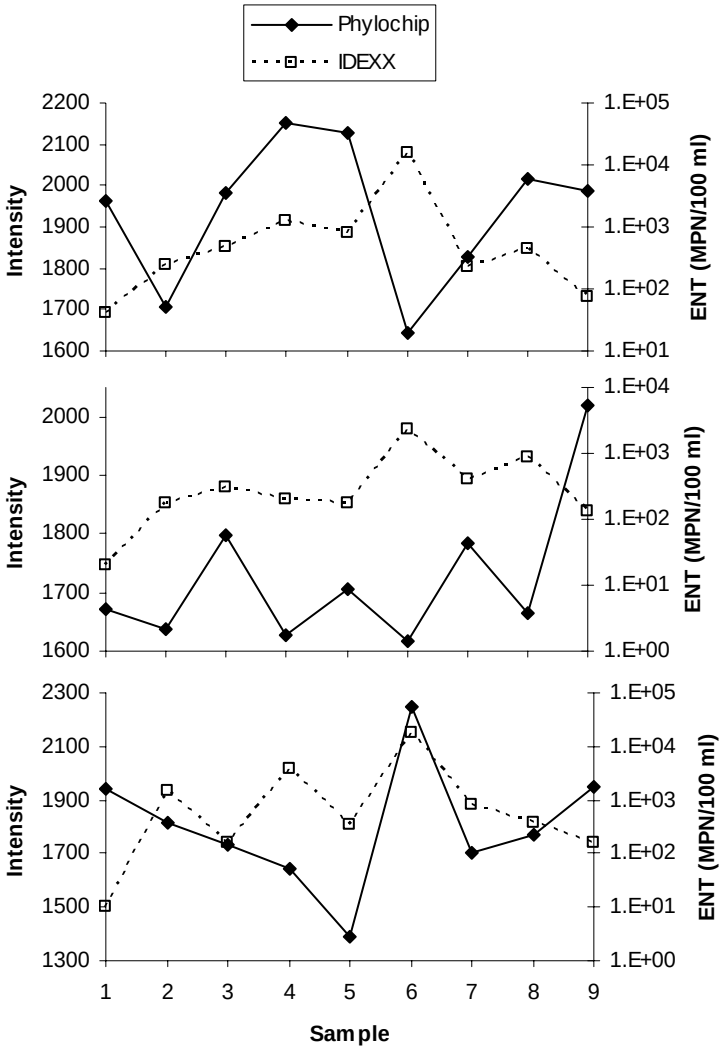

FIGURE 6-5: Comparison of *Enterococcus* spp. concentrations based on IDEXX assays and PhyloChip hybridization intensities.

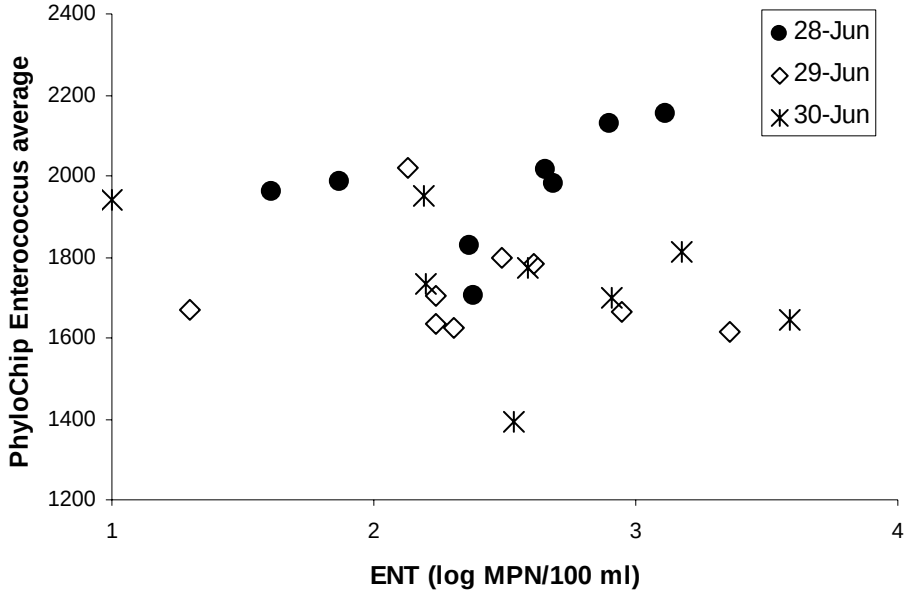

FIGURE 6-6: Scatterplot of *Enterococcus* spp. concentrations based on IDEXX assays (log-transformed concentrations, abscissa) and PhyloChip hybridization intensities (ordinate).

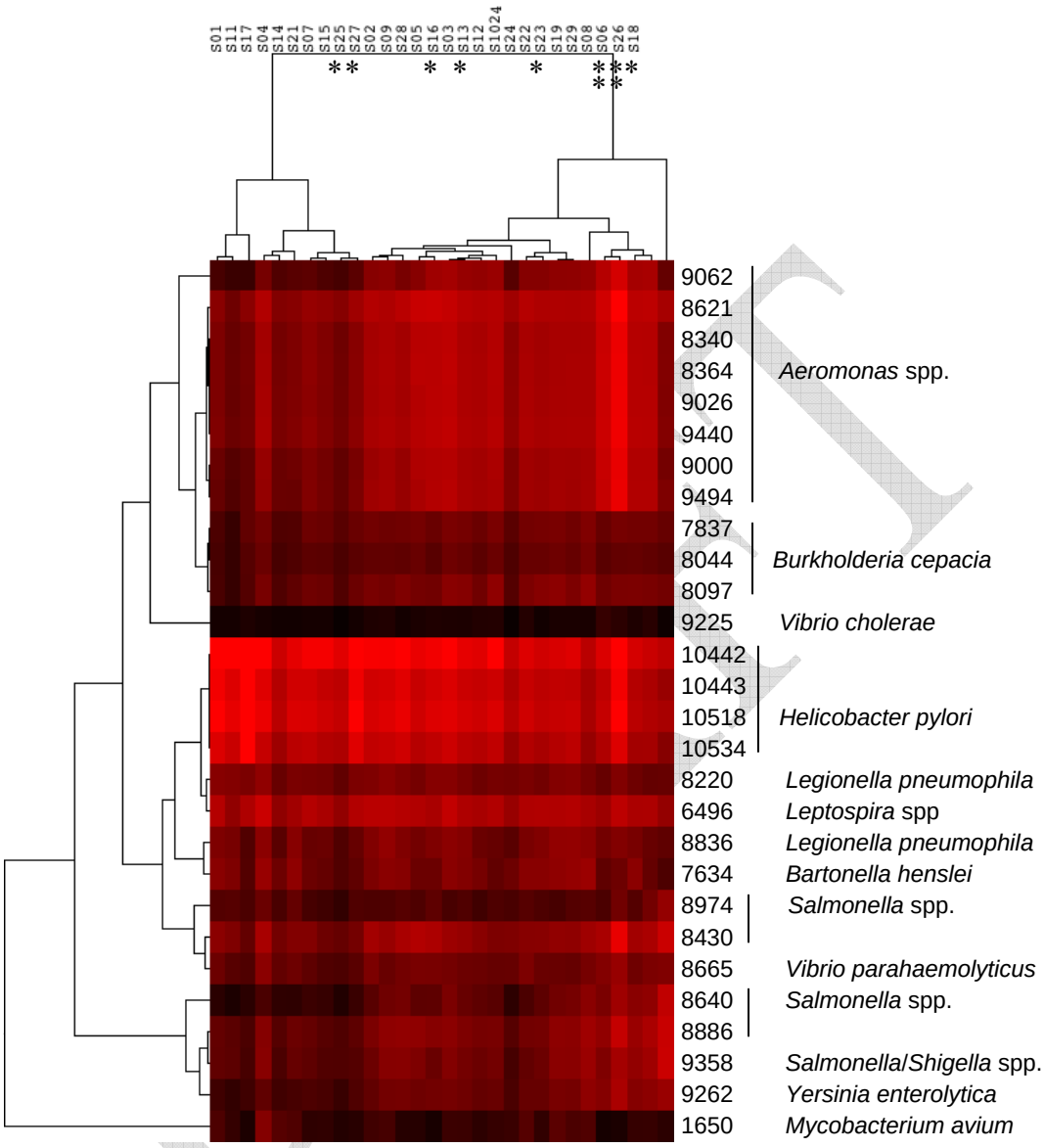

FIGURE 6-7: Hierarchical cluster analysis of all samples based on pathogen-containing OTUs, using the raw dataset. Pathogenic taxa are indicated on the right. The top clustering shows the sample clusters, the numbers on the right indicate the OTUs. Red intensities indicate the magnitude of the hybridization signal. The range of the human-specific *Bacteroides* concentrations are indicated for all samples (except human, S1024, sewage): * = 3.8 x 10^3 – 1.7 x 10^5 markers l^{-1}, ** = 4.2 x 10^6 – 1.5 x 10^7 markers l^{-1}, no mark = below detection limit.

Chapter 7: Microbiological Water Quality of Carrillo and Victoria Drains Discharge (Phase III Carrillo and Victoria Drain Sampling)

7.1 Introduction

Following the analysis of samples acquired in 2005 showing that both Haley and Hope Drains were discharging human waste markers, the City requested "snapshot" sampling of other drains that had historically high fecal indicator concentrations. In consultation with the City, two drains were selected and sampled. Importantly, this additional sampling was intended to provide a more comprehensive indication of how widespread drain contamination might be for the Mission Creek lower watershed.

7.2 Materials and Methods

Utilizing the same water sampling methods as before (see Chapter 2), initial water samples were taken from Carrillo and Victoria drains on August 3, 2006, along with a sample of the algae growing within the drainage from Victoria drain. A 3-day successive sampling of both drains occurred from September 19 – 22, 2006. Each drain was sampled once at approximately the same time of day. Samples were analyzed for FIB via IDEXX and human-specific *Bacteroides* markers via qPCR as previously described (see Chapter 2).

7.3 Results

Carrillo drain consistently had higher levels of *E. coli* and enterococci, while Victoria drain had higher total coliform levels on two of the sampling days (Table 7-1). The initial sampling of Carrillo drain on August 3, 2006 resulted in the highest number of human-specific *Bacteroides* markers. The 3-day sampling period resulted in one day of detection for each drain, but both sample concentrations were too near our limit of

quantification for the method, and only had targets amplify in one of nine analytical replicates (Table 7-2).

7.4 Conclusions

The high temporal variability in human-specific *Bacteroides* concentrations in Carrillo drain is similar to what we have seen for Haley and Hope drains (see Chapters 2 & 3). Future studies on Carrillo, Victoria, or any of the other storm drains in our system will need to address this variability with additional and more comprehensive sampling times.

TABLE 7-1: FIB results for Phase 3 Carrillo and Victoria drain survey. TC = total coliform, *E. coli* = *Escherichia coli*, Ent = enterococci (IDEXX).

ID	Description	TC (MPN/100 mL)	*E. coli* (MPN/100 mL)	Ent (MPN/100 mL)
803-3	Carrillo Drain	155310	5940	10760
803-4	Victoria Drain	>241960	3410	2880
803-5	algae from Victoria Drain		not analyzed	
919-01	Carrillo drain	198630	970	3890
919-02	Victoria drain	>241960	740	520
920-01	Carrillo drain	77010	850	5730
920-02	Victoria drain	>241960	410	1710
921-01	Carrillo drain	>241960	8800	>241960
921-02	Victoria drain	>241960	100	2660

TABLE 7-2: Human-specific *Bacteroides* qPCR results for Phase 3 Carrillo and Victoria drain survey. Average and SE values for water samples are targets/L, and targets/g wet for the algae sample. Number of replicates refers to analytical replicates (each sample was run in triplicate on every plate).

ID	Description	avg.targets/L (or g wet)	SE targets/L (or g wet)	# of replicates w/target	total # of replicates
803-3	Carrillo Drain	7.7E+05	1.8E+05	5	6
803-4	Victoria Drain	0.0E+00	0.0E+00	0	6
803-5	algae from Victoria Drain	0.0E+00	0.0E+00	0	6
919-01	Carrillo drain	4.5E+04	4.5E+04	1	9
919-02	Victoria drain	0.0E+00	0.0E+00	0	9
920-01	Carrillo drain	0.0E+00	0.0E+00	0	9
920-02	Victoria drain	1.2E+05	1.2E+05	1	9
921-01	Carrillo drain	0.0E+00	0.0E+00	0	9
921-02	Victoria drain	0.0E+00	0.0E+00	0	9

Chapter 8: Historical Analysis of City FIB Data for Lower Arroyo Burro and Mission Creeks

8.1 Introduction

Based on our short term 3-day snapshot studies in Phase II (see Chapter 2), we know that the FIB measurements via IDEXX contained high levels of variation from day to day, and little statistical significance between our sites in the Mission Creek and Arroyo Burro watersheds. Historical FIB data, made available by the City, was analyzed to determine if long term FIB results can better indicate fecal "hot spots" (i.e. urban drains).

8.2 Materials and Methods

Historical FIB test results from fourteen sampling spots in the Mission Creek watershed, and twenty-three spots in the Arroyo Burro watershed were obtained from the City of Santa Barbara. Of these sites, five in Mission Creek and eight in Arroyo Burro are similar to the sites we sampled during our 3-day snapshot studies (Tables 8-1 & 8-2). Sites that were only present in the City's data file for one sampling day (Victoria drain, MC above Victoria) were not included in the analysis. Samples labeled "MC @ Gutierrez" were added to the "Gutierrez" samples, and similarly "Mission Canyon Rd." samples were added to "MC @ Mission Canyon Rd.". Any sampling points labeled as "lab rep" were also removed, while points labeled "field rep" were kept.

We focused only on those *E. coli* (EC) and total enterococci (ENT) data points that were within range of the IDEXX tests, so no ">" or "<" data was included. Due to the large numbers of ">" data points for total coliform (TC) measurements, all TC measurements were excluded. The resulting data sets were analyzed separately by watershed, using all dates provided by the City (through September 2005) as well as

using only those data points corresponding to the AB411 dates (April 1 through October 31 of each sampled year through September 2005).

Data were imported in to SPSS version 12 (SPSS Inc., Chicago, IL), and error plots and mean plots were constructed, followed by homogeneity of variance tests and One-Way ANOVAs. Due to the significant and unequal variances of the means across the sites, the Dunnett's T3 pairwise comparison test was used in lieu of other analysis of variance post hoc tests which assume equal variance (i.e. Tukey's HSD).

8.3 Results

8.3.1 Mission Creek Watershed Analysis

- EC & ENT means (using all dates and the AB411 dates) were lowest for the "suburban" sites (12-14) and increased in the downstream "urban" sites, spiking to the highest means at site 7 (Carrillo drain) and site 5 (Haley drain) (Figs. 8-1 to 8-4).

- For EC using all dates, site 7 (Carrillo drain) was statistically significant from all sites except site 5 (Haley drain) (Table 8-3), but was not statistically different from any of the sites when only AB411 dates were used (Table 8-4).

- For ENT using all dates and AB411 dates, site 7 (Carrillo drain) and site 5 (Haley drain) were not significantly different from any sites (Tables 8-5 & 8-6).

8.3.2 Arroyo Burro Watershed Analysis

- In the Arroyo Burro watershed, EC & ENT means (using all dates) were highest at site 18 (Hope drain), site 16 (LPC head) and site 17 (AB below SRC) (Figs. 8-5 & 8-6). This trend was also the same for the EC means using the AB411 dates only (Fig. 8-7). For ENT means using the AB411 dates, the order was switched slightly so that the highest mean was at site 18 (Hope drain), followed by site 17 (AB below SRC), site 16 (LPC head), and site 15 (LPC above AB) (Fig. 8-8).

- For EC using all dates and AB411 dates, sites 18 (Hope drain), 16 (LPC head), 17 (AB below SRC) were not statistically different from any sites (Tables 8-7 & 8-8).

- For ENT using all dates, site 18 (Hope drain) was significantly different from sites 1 – 13 and sites 19 – 23, while sites 16 (LPC head) and 17 (AB below SRC) were not different from any sites (Table 9). Using the AB411 dates, a very different pattern emerged where site 18 (Hope drain) was not different from any sites (Table 10). Sites 17 (AB below SRC) and 15 (LPC above AB) were also not different from any sites, while site 16 (LPC head) was significantly different from sites 1 – 7, 10, 12 – 14, and 22 (Table 10).

8.4 Conclusions

There was a large difference in the number of statistically different sites depending on whether all dates were used, or only those during dry weather (AB411) dates. When AB411 dates were used, the number of significant sites was drastically reduced, and in one case (Arroyo Burro, ENT), the site most different from the others changed completely. Since it is well known that storms and storm related runoff can significantly alter bacterial community composition (see Chapter 4), it makes more sense to analyze the AB411 dates only.

Looking at whether or not these analyses on the AB411 dates can separate out the suspected "hot spot" urban drains, the result is mainly not for Mission Creek. Sites 5 (Haley drain), and 7 (Carrillo drain) were not different for either EC or ENT. However, site 11 (Westside drain) was significantly different for ENT from a single upstream "suburban" site (13 – Foothill), but was not different from any site for EC. There were also mixed results for urban drains in the Arroyo Burro watershed. Site 18 (Hope drain) was not different from any site for EC or ENT. However, site 8 (Mesa drain below culvert) was significantly different from five sites for EC (including an upstream

"suburban" site, and the site just above the culvert – site 9). For ENT, site 8 was significantly different from four sites (including AB lagoon mid, mouth and the ocean).

These results are similar to what we found from our 3-day snapshot studies. At Mission Creek, neither Haley drain nor Westside drain were significantly different from any site for EC or ENT (Carrillo drain was not part of that study). At Arroyo Burro, neither Mesa Creek (=Mesa drain below culvert) nor Hope drain were different from any site for EC or ENT.

TABLE 8-1: Mission Creek sites

Mission Creek

ID#	Site	Holden site ID (closest match)
1	MC lagoon	M4 (Mission lagoon)
2	MC2800 (=Montecito)	M5 (Montecito)
3	Gutierrez	
4	MC @ Haley	not equal to M7 (ours is above drain, the City's below)
5	Haley drain	M6 (Haley drain)
6	Carrillo	
7	Carrillo drain	
8	MC above OMC	
9	OMC above MC	M8 (OMC into MC)
10	Bohnett Park	
11	Westside drain	M9 (Westside drain)
12	MC @ Mission Canyon Rd.	
13	Foothill	
14	Rattlesnake	

TABLE 8-2: Arroyo Burro sites

Arroyo Burro

ID#	Site	Holden site ID (closest match)
1	surf (=AB ocean)	A1 (AB surf)
2	AB lagoon mouth @ depth	
3	AB lagoon mouth	A2 (AB lagoon mouth)
4	AB lagoon mid @ depth	
5	AB lagoon mid @ surface	
6	AB lagoon upper @ depth	
7	AB lagoon upper @ surface	A3 (above AB lagoon)
8	Mesa drain below culvert	A4 (Mesa Creek)
9	Mesa drain above culvert	
10	AB1850 (=ABC @ Cliff Dr.)	A5 (AB Creek @ Cliff Dr.)
11	AB below LPC	A6 (AB below Las Positas Creek)
12	Portesuelo (=Veronica Springs)	
13	AB above LPC	
14	Valle Verde	
15	LPC above AB	A10 (Las Positas Creek @ Modoc)
16	LPC head	
17	AB below SRC	
18	Hope drain	A9 (Hope drain)
19	AB above SRC	
20	SRC above AB	
21	SRC Foothill	
22	Jesusita	
23	Barger	

TABLE 8-3: Post-hoc testing results of the Mission Creek EC samples (all dates). Numbers in the last column for each sample row indicate sites significantly different from that site.

Mission Creek Dunnett's T3 (alpha = 0.05)

ID#	Site	Dates	# of EC	EC significant difference
1	MC lagoon	8/9/04-9/26/05	74	14, 13, 12, 7, 6
2	MC2800 (=Montecito)	6/4/01-9/26/05	313	14, 13, 12, 7
3	Gutierrez	6/5/01-9/7/05	97	7
4	MC @ Haley	6/5/01-3/20/02	39	14, 13, 12, 7, 6
5	Haley drain	6/19/01-10/15/03	38	
6	Carrillo	6/5/01-10/26/04	93	14, 12, 7, 4, 1
7	Carrillo drain	8/13/02-9/6/05	36	14, 13, 12, 11, 10, 9, 8, 6, 4, 3, 2, 1
8	MC above OMC	6/12/01-10/26/04	43	7
9	OMC above MC	6/12/01-10/26/04	103	7
10	Bohnett Park	6/5/01-9/26/05	173	14, 13, 12, 7
11	Westside drain	6/12/01-9/26/05	176	14, 13, 12, 7
12	MC @ Mission Canyon Rd.	7/31/01-8/16/05	78	11, 10, 7, 6, 4, 2, 1
13	Foothill	6/5/01-9/14/04	59	11, 10, 7, 4, 2, 1
14	Rattlesnake	7/31/01-8/16/05	81	11, 10, 7, 6, 4, 2, 1

TABLE 8-4: Post-hoc testing results of the Mission Creek EC samples (AB411 dates). Numbers in the last column for each sample row indicate sites significantly different from that site.

Mission Creek		AB411		Dunnett's T3 (alpha = 0.05)
ID#	**Site**	**Dates**	**# of EC**	**EC significant difference**
1	MC lagoon	8/9/04-9/26/05	41	14, 13, 12
2	MC2800 (=Montecito)	6/4/01-9/26/05	176	14, 13, 12
3	Gutierrez	6/5/01-9/7/05	66	
4	MC @ Haley	6/5/01-10/30/01	21	
5	Haley drain	6/19/01-10/15/03	21	
6	Carrillo	6/5/01-10/26/04	59	
7	Carrillo drain	8/13/02-9/6/05	25	
8	MC above OMC	6/12/01-10/26/04	16	
9	OMC above MC	6/12/01-10/26/04	67	
10	Bohnett Park	6/5/01-9/26/05	102	
11	Westside drain	6/12/01-9/26/05	102	
12	MC @ Mission Canyon Rd.	7/31/01-8/16/05	44	2, 1
13	Foothill	6/5/01-9/14/04	32	2, 1
14	Rattlesnake	7/31/01-8/16/05	49	2, 1

TABLE 8-5: Post-hoc testing results of the Mission Creek ENT samples (all dates). Numbers in the last column for each sample row indicate sites significantly different from that site.

Mission Creek Dunnett's T3 (alpha = 0.05)

ID#	Site	Dates	# of ENT	ENT significant difference
1	MC lagoon	8/9/04-9/26/05	75	13, 12
2	MC2800 (=Montecito)	6/4/01-9/26/05	309	13, 12
3	Gutierrez	6/5/01-9/7/05	96	13, 12
4	MC @ Haley	6/5/01-3/20/02	37	
5	Haley drain	6/19/01-10/15/03	32	
6	Carrillo	6/5/01-10/26/04	92	
7	Carrillo drain	8/13/02-9/6/05	43	
8	MC above OMC	6/12/01-10/26/04	40	
9	OMC above MC	6/12/01-10/26/04	97	13, 12
10	Bohnett Park	6/5/01-9/26/05	169	14, 13, 12
11	Westside drain	6/12/01-9/26/05	173	14, 13, 12
12	MC @ Mission Canyon Rd.	7/31/01-8/16/05	79	11, 10, 9, 3, 2, 1
13	Foothill	6/5/01-9/14/04	60	11, 10, 9, 3, 2, 1
14	Rattlesnake	7/31/01-8/16/05	83	11, 10

TABLE 8-6: Post-hoc testing results of the Mission Creek ENT samples (AB411 dates). Numbers in the last column for each sample row indicate sites significantly different from that site.

Mission Creek		AB411		Dunnett's T3 (alpha = 0.05)
ID#	**Site**	**Dates**	**# of ENT**	**ENT significant difference**
1	MC lagoon	8/9/04-9/26/05	42	
2	MC2800 (=Montecito)	6/4/01-9/26/05	173	
3	Gutierrez	6/5/01-9/7/05	64	13, 12
4	MC @ Haley	6/5/01-10/30/01	17	
5	Haley drain	6/19/01-10/15/03	14	
6	Carrillo	6/5/01-10/26/04	58	
7	Carrillo drain	8/13/02-9/6/05	30	
8	MC above OMC	6/12/01-10/26/04	12	
9	OMC above MC	6/12/01-10/26/04	61	13, 12
10	Bohnett Park	6/5/01-9/26/05	97	
11	Westside drain	6/12/01-9/26/05	98	13
12	MC @ Mission Canyon Rd.	7/31/01-8/16/05	44	9, 3
13	Foothill	6/5/01-9/14/04	33	11, 9, 3
14	Rattlesnake	7/31/01-8/16/05	50	

TABLE 8-7: Post-hoc testing results of the Arroyo Burro EC samples (all dates). Numbers in the last column for each sample row indicate sites significantly different from that site.

Arroyo Burro Dunnett's T3 (alpha = 0.05)

ID#	Site	Dates	# of EC	EC significant difference
1	surf (=AB ocean)	5/26/04-9/27/05	82	23, 9, 8, 2
2	AB lagoon mouth @ depth	5/26/04-11/1/04	26	12, 1
3	AB lagoon mouth	5/26/04-9/27/05	95	
4	AB lagoon mid @ depth	5/26/04-5/23/05	32	
5	AB lagoon mid @ surface	5/26/04-5/23/05	32	
6	AB lagoon upper @ depth	5/26/04-5/23/05	29	
7	AB lagoon upper @ surface	5/26/04-5/23/05	31	
8	Mesa drain below culvert	9/10/01-9/27/05	162	22, 12, 9, 1
9	Mesa drain above culvert	8/30/04-9/27/05	57	8, 1
10	AB1850 (=ABC @ Cliff Dr.)	5/21/01-9/27/05	350	
11	AB below LPC	7/3/01-11/2/04	96	
12	Portesuelo (=Veronica Springs)	7/3/01-4/23/03	51	23, 8, 2
13	AB above LPC	7/3/01-2/1/05	86	
14	Valle Verde	5/23/01-9/20/05	108	
15	LPC above AB	7/3/01-11/2/04	89	
16	LPC head	5/23/01-9/19/05	91	
17	AB below SRC	5/23/01-9/20/05	91	
18	Hope drain	6/13/01-9/20/05	53	
19	AB above SRC	5/23/01-11/2/04	69	
20	SRC above AB	5/23/01-11/2/04	73	
21	SRC Foothill	5/23/01-2/13/02	19	
22	Jesusita	8/15/01-9/12/05	62	8
23	Barger	5/23/01-9/27/05	96	12, 1

TABLE 8-8: Post-hoc testing results of the Arroyo Burro EC samples (AB411 dates). Numbers in the last column for each sample row indicate sites significantly different from that site

Arroyo Burro				Dunnett's T3 (alpha = 0.05)
ID#	**Site**	**Dates**	**# of EC**	**EC significant difference**
1	surf (=AB ocean)	5/26/04-9/27/05	51	23, 8, 2
2	AB lagoon mouth @ depth	5/26/04-10/25/04	25	1
3	AB lagoon mouth	5/26/04-9/27/05	61	
4	AB lagoon mid @ depth	5/26/04-5/23/05	31	
5	AB lagoon mid @ surface	5/26/04-5/23/05	31	
6	AB lagoon upper @ depth	5/26/04-5/23/05	28	
7	AB lagoon upper @ surface	5/26/04-5/23/05	30	
8	Mesa drain below culvert	9/10/01-9/27/05	90	22, 20, 12, 9, 1
9	Mesa drain above culvert	8/30/04-9/27/05	27	8
10	AB1850 (=ABC @ Cliff Dr.)	5/21/01-9/27/05	214	
11	AB below LPC	7/3/01-10/5/04	57	
12	Portesuelo (=Veronica Springs)	7/3/01-4/23/03	34	8
13	AB above LPC	7/3/01-10/5/04	50	
14	Valle Verde	5/23/01-9/20/05	71	
15	LPC above AB	7/3/01-10/5/04	51	
16	LPC head	5/23/01-9/19/05	51	
17	AB below SRC	5/23/01-9/20/05	54	
18	Hope drain	6/13/01-9/20/05	32	
19	AB above SRC	5/23/01-10/5/04	38	
20	SRC above AB	5/23/01-10/5/04	42	8
21	SRC Foothill	5/23/01-10/31/01	12	
22	Jesusita	8/15/01-9/12/05	34	8
23	Barger	5/23/01-9/27/05	60	1

TABLE 8-9: Post-hoc testing results of the Arroyo Burro ENT samples (all dates). Numbers in the last column for each sample row indicate sites significantly different from that site.

Arroyo Burro ID#	Site	Dates	# of ENT	Dunnett's T3 (alpha = 0.05) ENT significant difference
1	surf (=AB ocean)	5/26/04-9/27/05	66	23, 18, 10, 8
2	AB lagoon mouth @ depth	5/26/04-11/1/04	16	23, 18, 10, 8
3	AB lagoon mouth	5/26/04-9/27/05	90	18, 8, 4
4	AB lagoon mid @ depth	5/26/04-5/23/05	21	23, 18, 10, 8, 7, 3
5	AB lagoon mid @ surface	5/26/04-5/23/05	28	23, 18, 10, 8
6	AB lagoon upper @ depth	5/26/04-5/23/05	27	23, 18, 10, 8
7	AB lagoon upper @ surface	5/26/04-5/23/05	30	23, 18, 10, 8, 4
8	Mesa drain below culvert	9/10/01-9/27/05	158	18, 12, 7, 6, 5, 4, 3, 2, 1
9	Mesa drain above culvert	8/30/04-9/27/05	58	18
10	AB1850 (=ABC @ Cliff Dr.)	5/21/01-9/27/05	343	18, 12, 7, 6, 5, 4, 2, 1
11	AB below LPC	7/3/01-11/2/04	95	18
12	Portesuelo (=Veronica Springs)	7/3/01-4/23/03	69	23, 18, 10, 8
13	AB above LPC	7/3/01-2/1/05	86	18
14	Valle Verde	5/23/01-9/20/05	108	
15	LPC above AB	7/3/01-11/2/04	92	
16	LPC head	5/23/01-9/19/05	93	
17	AB below SRC	5/23/01-9/20/05	93	
18	Hope drain	6/13/01-9/20/05	56	23, 22, 21, 20, 19, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1
19	AB above SRC	5/23/01-11/2/04	70	18
20	SRC above AB	5/23/01-11/2/04	88	18
21	SRC Foothill	5/23/01-2/13/02	21	18
22	Jesusita	8/15/01-9/12/05	70	18
23	Barger	5/23/01-9/27/05	93	18, 12, 7, 6, 5, 4, 2, 1

TABLE 8-10: Post-hoc testing results of the Arroyo Burro ENT samples (AB411 dates). Numbers in the last column for each sample row indicate sites significantly different from that site

Arroyo Burro				Dunnett's T3 (alpha = 0.05)
ID#	**Site**	**Dates**	**# of ENT**	**ENT significant difference**
1	surf (=AB ocean)	5/26/04-9/27/05	37	16, 8
2	AB lagoon mouth @ depth	5/26/04-10/25/04	15	16, 10, 8
3	AB lagoon mouth	5/26/04-9/27/05	58	16
4	AB lagoon mid @ depth	5/26/04-5/23/05	20	16, 13, 10, 8
5	AB lagoon mid @ surface	5/26/04-5/23/05	27	16, 8
6	AB lagoon upper @ depth	5/26/04-5/23/05	26	16
7	AB lagoon upper @ surface	5/26/04-5/23/05	29	16
8	Mesa drain below culvert	9/10/01-9/27/05	87	5, 4, 2, 1
9	Mesa drain above culvert	8/30/04-9/27/05	28	
10	AB1850 (=ABC @ Cliff Dr.)	5/21/01-9/27/05	210	16, 4, 2
11	AB below LPC	7/3/01-10/5/04	56	
12	Portesuelo (=Veronica Springs)	7/3/01-4/23/03	41	16
13	AB above LPC	7/3/01-10/5/04	49	16, 4
14	Valle Verde	5/23/01-9/20/05	68	16
15	LPC above AB	7/3/01-10/5/04	53	
16	LPC head	5/23/01-9/19/05	51	22, 14, 13, 12, 10, 7, 6, 5, 4, 3, 2, 1
17	AB below SRC	5/23/01-9/20/05	53	
18	Hope drain	6/13/01-9/20/05	28	
19	AB above SRC	5/23/01-10/5/04	39	
20	SRC above AB	5/23/01-10/5/04	53	
21	SRC Foothill	5/23/01-10/31/01	12	
22	Jesusita	8/15/01-9/12/05	39	16
23	Barger	5/23/01-9/27/05	57	

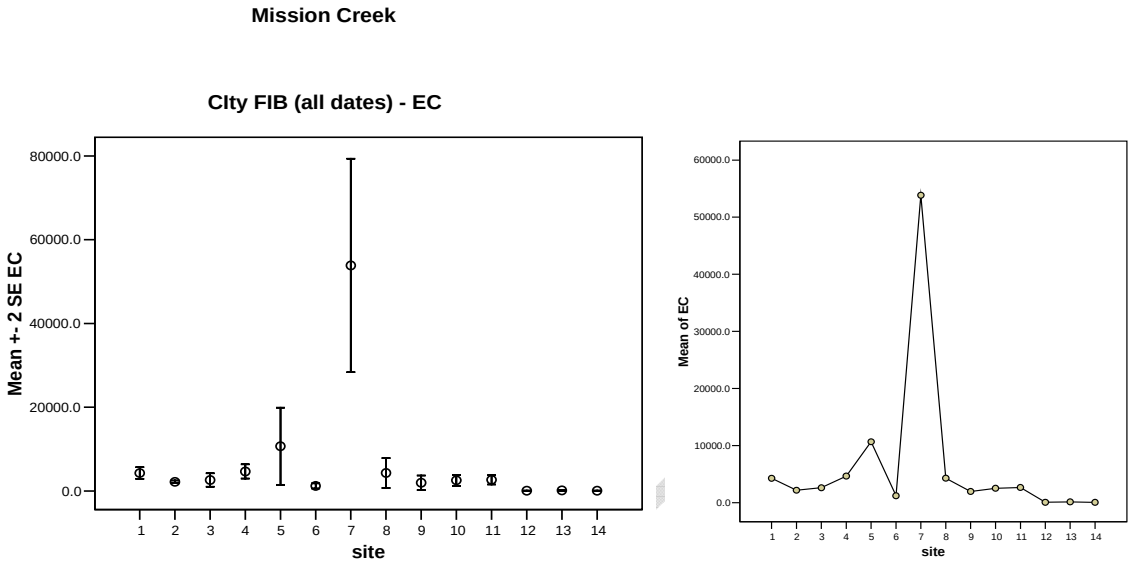

FIGURE 8-1: Error plot (left) and means plot (right) of the EC samples in the Mission Creek watershed (all dates), illustrating the significant different in the variances and means across the sites (P < 0.001).

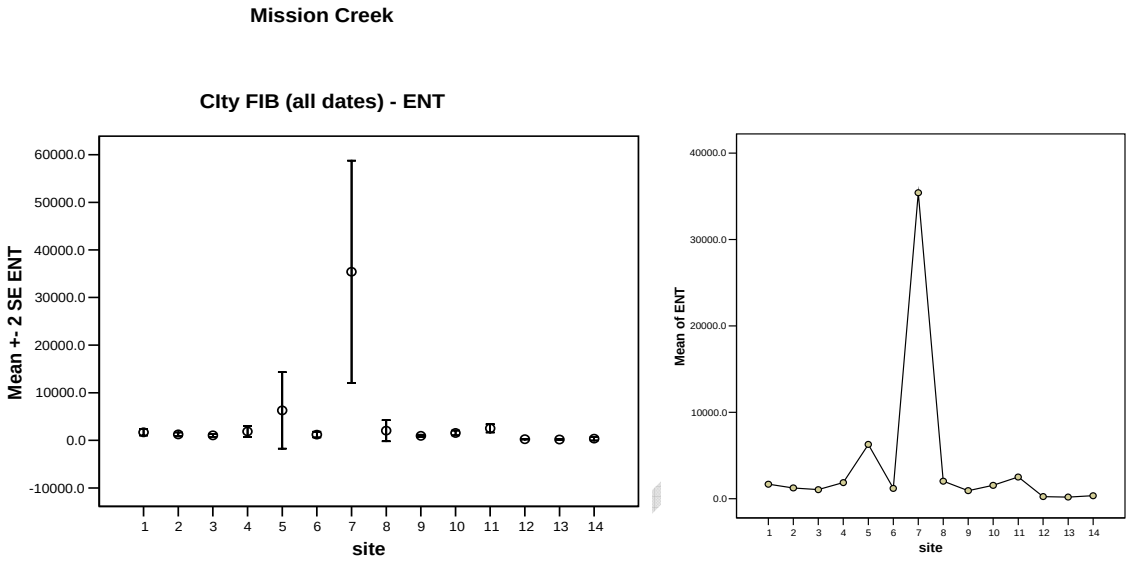

FIGURE 8-2: Error plot (left) and means plot (right) of the ENT samples in the Mission Creek watershed (all dates), illustrating the significant different in the variances and means across the sites (P < 0.001).

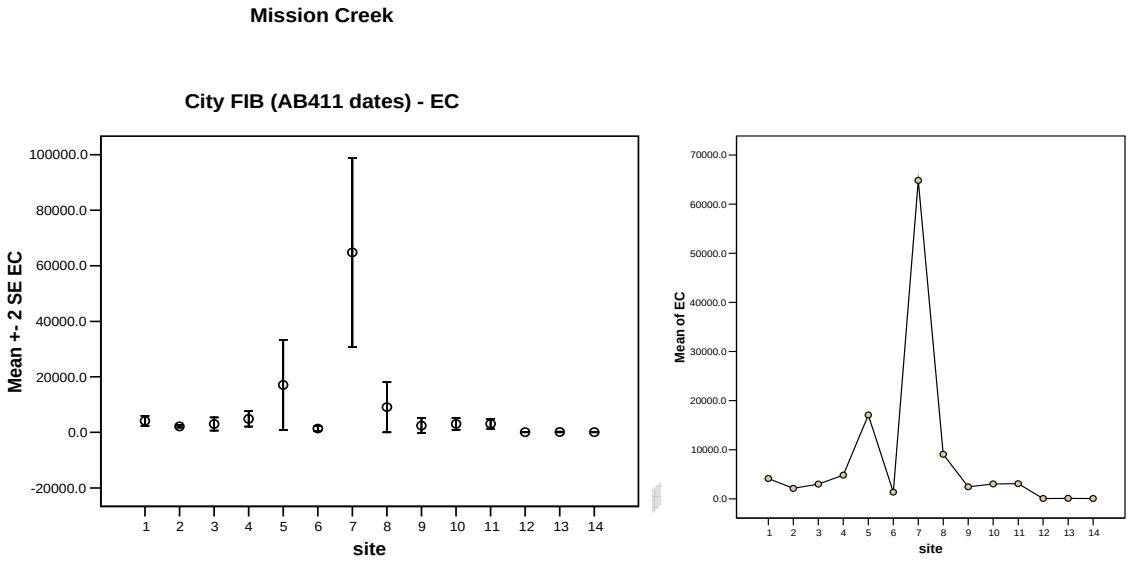

FIGURE 8-3: Error plot (left) and means plot (right) of the EC samples in the Mission Creek watershed (AB411 dates only), illustrating the significant different in the variances and means across the sites (P < 0.001).

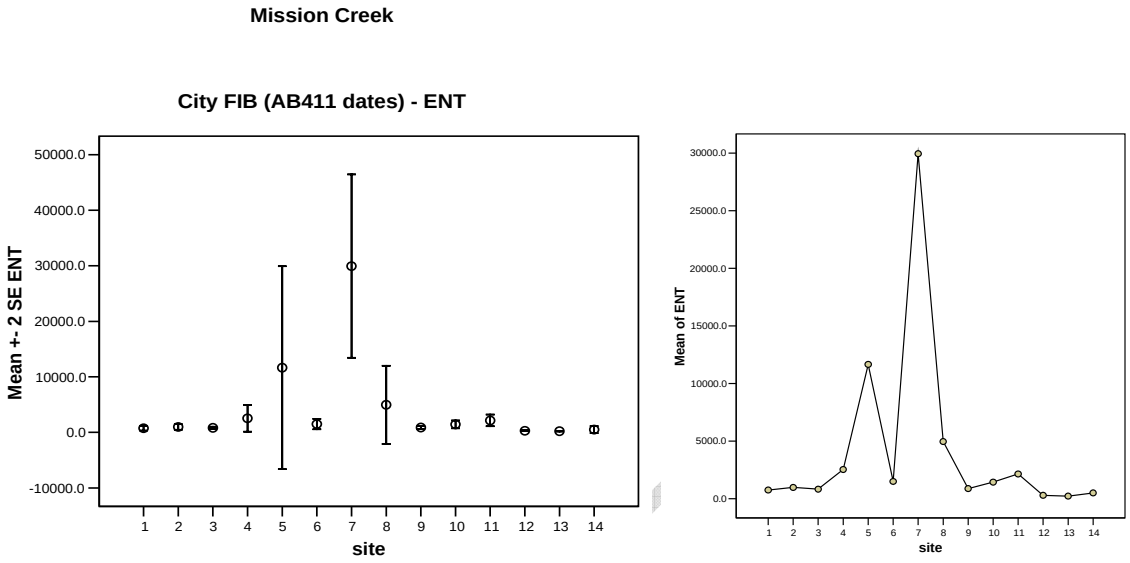

Figure 8-4: Error plot (left) and means plot (right) of the ENT samples in the Mission Creek watershed (AB411 dates only), illustrating the significant different in the variances and means across the sites (P < 0.001).

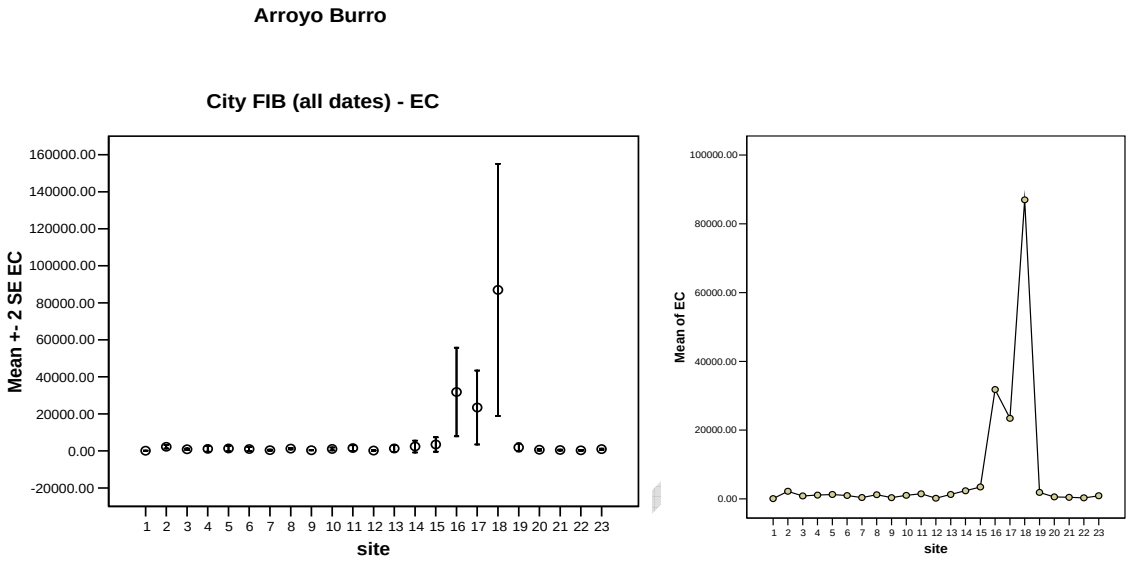

FIGURE 8-5: Error plot (left) and means plot (right) of the EC samples in the Arroyo Burro watershed (all dates), illustrating the significant different in the variances and means across the sites (P < 0.001).

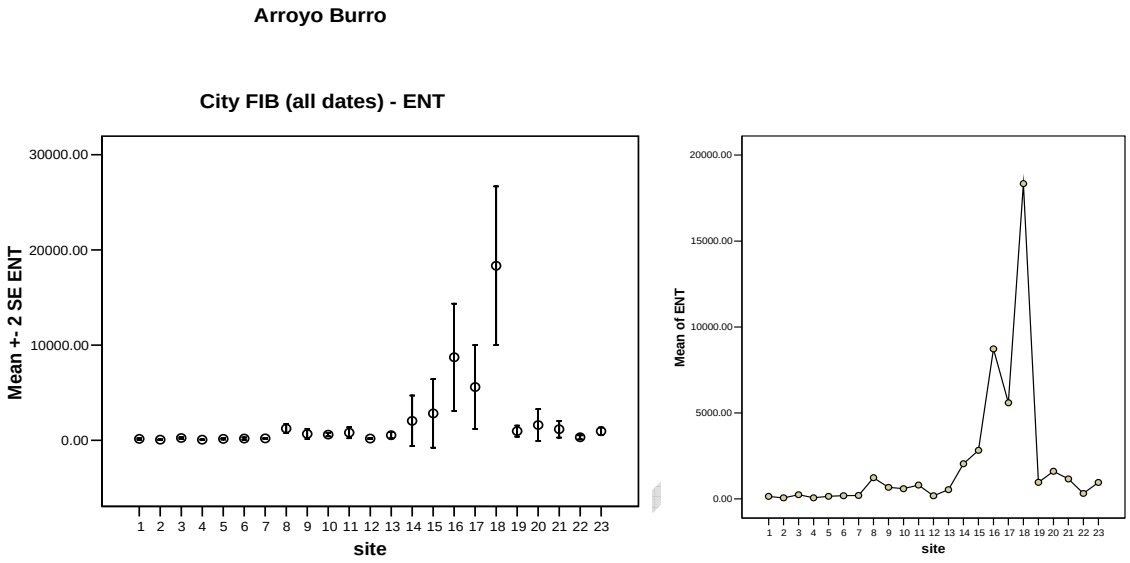

FIGURE 8-6: Error plot (left) and means plot (right) of the ENT samples in the Arroyo Burro watershed (all dates), illustrating the significant different in the variances and means across the sites (P < 0.001).

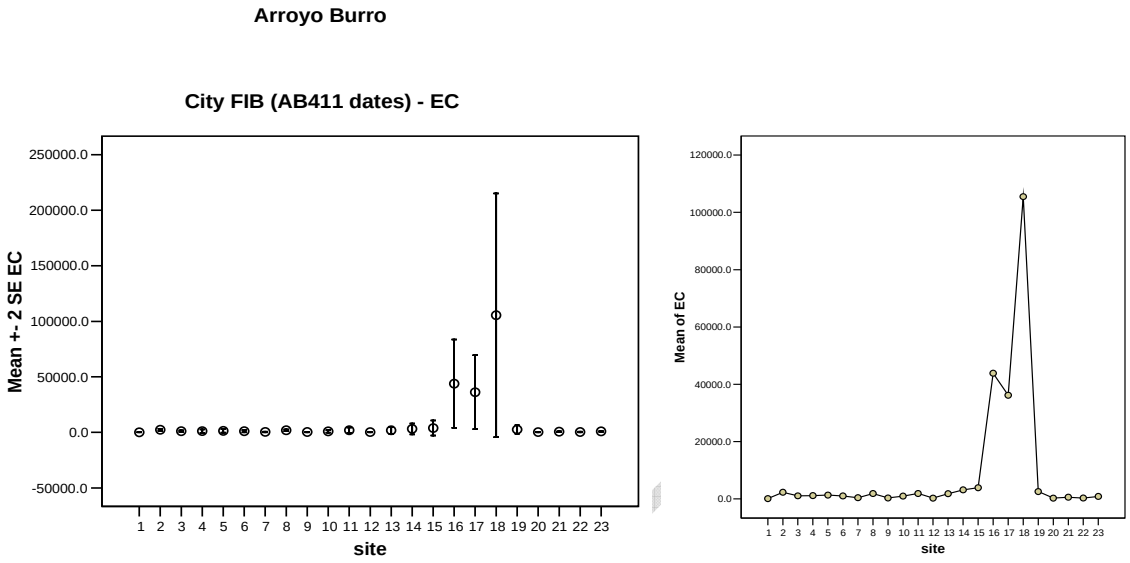

FIGURE 8-7: Error plot (left) and means plot (right) of the EC samples in the Arroyo Burro watershed (AB411 dates only), illustrating the significant different in the variances and means across the sites (P < 0.001).

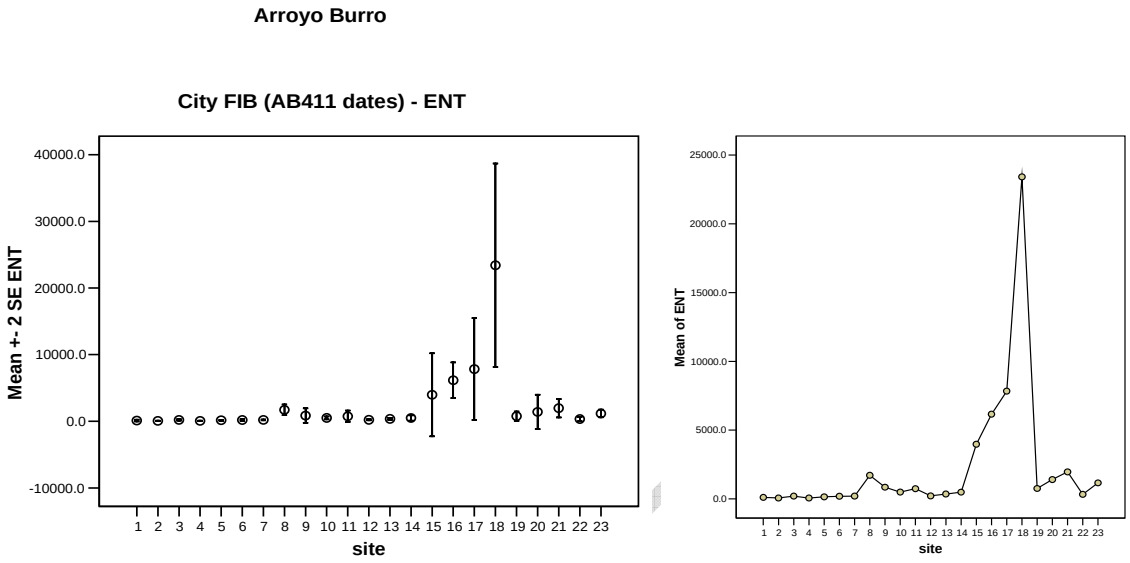

FIGURE 8-8: Error plot (left) and means plot (right) of the ENT samples in the Arroyo Burro watershed (AB411 dates only), illustrating the significant different in the variances and means across the sites (P < 0.001).

Appendices

A digital copy of this report as well as data generated and used in this report are saved to the appended CD.

Observations in Macro and Micro algae Contributions to Bacteria Populations and Implications for Beach Advisories

Steve Peters, Water Quality Specialist
Santa Cruz County Environmental Health Services
November 2006

Background: Since 2004 Santa Cruz County EHS has noticed that both Capitola Beach and Cowell Beach have been posted with Water Quality Advisories the first or second week in June with no obvious cause. In the past we have noticed large flocks of birds and have always blamed the beach postings on large amounts of bird droppings found when the birds are present. Many times there was no obvious bird presence nor has there been any sewage discharges to account for the increase in bacteria levels.

The County of Santa Cruz Environmental Health Services conducted tests on kelp found in the tidal zone at several Santa Cruz County beaches to determine if decaying kelp could be one of the sources of elevated fecal indicator bacteria that has caused Capitola and Cowell Beaches to be posted with swimming advisories.

In 2005 kelp samples were taken on June 27, July 6, July 7, July 11, July 19, and August 1. In 2006 kelp samples were taken a single time on June 26. In addition, samples were taken and analyzed from sand at various depths to ground water and also from a tidal impoundment at Santa Cruz Main Beach.

Sample results from the tidal impoundment indicated that there was an additional source of bacteria as the pond had sat in the sun for a period of time and there was no evidence of bird contribution since there were no droppings in the water or bird feathers along the edges.

This led EHS to seek another source of elevated bacteria and led us to analyze micro algae since there had been increased observations of red tide in the area.

Materials and Methods

Kelp samples were collected and put into sterile Nasco Whirl-Pak sample bags from Capitola Beach, Hooper's Beach, the beach at 38th Avenue, Cowell Beach, Mitchell's Cove Beach, Natural Bridges Beach, and Waddell Beach.

Sand samples were extracted from a hole dug to the water level and collected from 3", 6", and 12". Samples were taken from Schwan Lake, San Lorenzo River mouth, Neary Lagoon, Soquel Creek mouth, and Aptos Creek mouth. These sites were chosen due to proximity to ocean sample sites that are periodically posted with water quality advisories. These samples were also collected into Nasco Whirl-Pak sample bags using sterile tongue depressors.

Micro algae samples were taken using a plankton net that was dropped from Santa Cruz Municipal Pier, Capitola Pier, and Seacliff Cement Ship Pier. Samples were placed into Nasco Whirl Pak bags and sub-samples were sent to The California State Department of Health State Mussel Watch Program for identification to species.

After returning to the Public Health Lab a small amount of sterile water was added to each bag containing a small amount of kelp or sand. The sample was then mashed to break it up and the bag was filled to the 100ml. mark. Water from each bag was then analyzed using Idexx Colilert-18, Idexx Enterolert, or membrane-filtration for fecal coliform bacteria. Water from plankton tows and the sample tidal pond sample were analyzed after shaking the sample bag.

The bags were sealed and left to sit at room temperature in the Public Health Lab for twenty-four hours and re-tested using the same type of analysis done initially, Idexx Colilert-18, Idexx Enterolert, or membrane-filtration for fecal coliform bacteria. In some cases water was again tested after sitting at room temperature for a total of 48 hours.

Test results in all cases showed low levels of fecal indicator bacteria at initial testing and extremely high levels of bacteria as determined by Idexx Colilert-18 and membrane-filtration for fecal coliform bacteria. Idexx Enterolert analysis showed variable results. Waddell Beach results showed no bacteria initially or after 24 hours. Hooper's Beach, 38th Avenue Beach, and Waddell Beach were tested one time each for the 2005 samplings. Cowell Beach and Capitola Beach were the only sites sampled for kelp in 2006.

The sample from the tidal pond was not analyzed after the initial analysis.

Results are presented in the table below. Where there is a third set of numbers in the column it indicates that the sample was held for analysis for 48 hours after the initial analysis.

Several samples were tested to species from colonies tested for fecal coliform bacteria and extracted from positive E. coli results in Idexx Colilert-18 testing. Organisms found were:

E. coli
Serratia rubidaea
Klebsiella oxytaca
Klebsiella pneumoniae
Vibrio alginolyticus

Sample results are shown below. All sample results are expressed as MPN/100 mls. sample.

2005

Date/Location/ (Sample type)	**E.coli (0/24hrs)**	**Total Coli(0/24hrs)**	**Entero (0/24hrs)**	**Fecal Coli (0/24hrs)**
27June/Capitola Beach	no sample	no sample	no sample	pos/TNTC
/ Cowell Beach	no sample	no sample	no sample	pos/TNTC
/ Hooper's Beach	no sample	no sample	no sample	pos/TNTC
/Mitchell's Cove	no sample	no sample	no sample	pos/TNTC
6July/Capitola Beach	5 / >24192	5 / >24192	5 / >24192	no sample
/ 38th Ave.	5 / >24192	10 / >24192	5 / >24192	no sample
/ Cowell Beach	5 / >24192	10 / >24192	5 / 5	no sample
/Mitchell's Cove	5 / >24192	5 / >24192	5 / 933	no sample
/ Natural Bridges	5 / >24192	30 / >24192	5 / 5	no sample
/ Waddell Beach	5 / 5	5 / 5	5 / 5	no sample
11July/ Capitola	31 / >24192	98 / >24192	5 / 3076	no sample
19July/ Capitola	5 / >24192	5 / >24192	5 / 5	no sample
/ Cowell Beach	10 / >24192	31 / >24192	5 / 379	no sample
/ Mitchell's Cove	5 / >24192	31 / >24192	5 / 5	no sample
/ Natural Bridges	5 / >24192	5 / >24192	5 / 467	no sample
1Aug/Capitola/**H2O**	52/86	181/161	10/5	no sample
/Capitola/**kelp**	10/>24192	20/>24192	5/425	no sample
/Hooper's/**H2O**	171/132	594/256	5/5	no sample
/Hooper's/**kelp**	5/1956	5/2046	5/5	no sample
/38th/**H2O**	5/5	5/5	5/5	no sample
/38th/**kelp**	5/5/146	10/>24192/>24192	5/4352	no sample
/Cowell/**H2O**	52/63	201/85	10/5	no sample
/Cowell/**kelp**	20/24192	20/>24192	5/>24192	no sample
/Mitchell's/**H2O**	5/5	10/5	5/5	no sample
/Mitchell's/**kelp**	5/>24192	5/>24192	5/5	no sample
/Nat. Bridges/**H2O**	5/5	5/5	5/5	no sample
/ Nat. Bridges/**kelp**	5/5/5	5/>24192/>24192	5/5	no sample
/Waddell/**H2O**	5/5	5/5	5/5	no sample
/ Waddell/**kelp**	5/5/5	5 / >24192/>24192	5 / 5	no sample

2006

Date/Location/ (Sample type)	**E.coli (0/24hrs)**	**Total Coli(0/24hrs)**	**Entero (0/24hrs**	**Fecal Coli (0/24hrs)**
6 June/Cowell/**kelp**	228/2187	336/>24192	10/<5	no sample
/Cowell	61/>24192	130/>24192	31/>24192	no sample
/Cowell	<5/>24192	109/>24192	20/4611	no sample
/Capitola	<5/>24192	<5/>24192	<5/108	no sample
/Capitola	10/>24192	132/>24192	<5/52	no sample
/Capitola	74/>24192	529/>24192	10/24192	no sample
3 Oct./SC Wharf/**micro**	<5/20/<5	85/2603/1956	<5/<5/<5	no sample
/Capitola Pier	62/399/602	241/2909/3654	<5/>24192/>24192	no sample
/Seacliff Pier	<5/41/10	10/1355/1355	<5/>24192/>24192	no sample
24 Oct/SC Wharf	52/24192/19863	63/24192/24192	<5/10/>24192	no sample
/Capitola Pier	20/3624/2489	85/5794/2723	<5/<5/>24192	no sample
/Seacliff Pier	146/63/10	243/74/20	<5/10/30	no sample
31 Oct/SC Wharf	10/20/<5	10/73/193	<5/<5/<5	no sample
/Capitola Pier	20/31/158	98/41/195	<5/<5/<5	no sample
/Seacliff Pier	<5/<5/<5	<5/<5/<5	<5/<5/<5	no sample
29 June/ Tidal Pool	3255	14136	613	no sample
5 June/Schwan-3”/**sand**	20/<10/20	82/126/2481	242/452/73	no sample
/Schwan-6”	40/<10/10	60/20/1650	40/194/108	no sample
/Schwan-12”	20/40/884	40/126/>24192	126/60/121	no sample
/SLR Mouth-3”	20/40/63	148/104/201	<10/20/20	no sample
/SLR Mouth-6”	20/<10/350	20/40/1162	<10/<10/<5	no sample
/SLR Mouth-12”	<10/<10/379	<10/20/6488	<10/<10/<5	no sample
/Neary- 3”	<10/<10/379	<10/20/6488	<10/<10/<5	no sample
/Neary-6”	<10/62/6867	<10/62/10462	<10/20/20	no sample
/Neary-12”	<10/<10/2247	<10/<10/17329	<10/<10/<5	no sample
6 June/Aptos Cr-3”	194/703	218/839	<10/<5	no sample
/Aptos Cr-6”	62/97	126/228	<10/<5	no sample
/Aptos Cr-12”	20/52	104/120	<10/<5	no sample
/Soquel Cr-3”	40/10	82/187	20/31	no sample
/Soquel Cr-6”	60/52	126/187	<10/20	no sample
/Soquel Cr- 12”	104/74	242/313	82/20	no sample

2007

27 Nov/Red Tide	**E.coli (0/24/48hrs**)	**Total Coli(0/24/48hrs)**	**Entero (0/24/48hrs**	**Fecal Coli (0/24hrs)**
/ Capitola Pier	109/52/41	243/74/41	<5/<5/<5	no sample
/ SC Wharf	30/<5/<5	880/25000/25000	<5/<5/<5	no sample
/ Seacliff Pier	85/2143/3255	738/25000/25000	<5/<5/<5	no sample

Results: Both macro and micro algae results indicate that there is an increase in bacteria over time and can be influenced by both increased temperature and lack of circulation at public beaches. In the case of macro algae samples it was noticed that prior to large amounts of kelp on local beaches there was a significant swell that ripped up kelp that was then left to rot on the beach. In 2006 a small segment of Cowell beach was posted with Water Quality Advisories for 51 days due to a large block of kelp that was left to rot under the sand. Current beach management requirements do not allow the removal of kelp that is below the high tide line.

Red tide was suggested as a possible source of elevated bacteria due to a tidal pool developing with a high tide in an area known to have a bloom of red tide. Bacteria results for micro algae suggest that may also contribute to the elevation of bacteria in water samples as the bloom crashes and decomposes. Again, poor water circulation in an area can contribute to the increases of bacteria and the posting of Water Quality Advisories. Micro algae blooms have the added problem of characteristic odors that are offensive to some people. Various red tide organisms have odors that are species specific and some may be pleasant while others are extremely offensive.

Bacteria in sand samples grew much more slowly than did the macro and micro algae samples. Results indicate that bacteria do inhabit sand but the sand may have an inhibitory affect on the bacteria as well as a filtering affect for organic components.

Discussion: Results from the past two years of studies indicate that much of the bacteria that cause beach postings can come from natural sources. Since the State of California has the potential to experience heavy red tides in some areas this may explain elevated bacteria that has caused the posting of Water Quality Advisories independent of any other sources. Rotting kelp, while providing some nurturing for organisms at the lower level of the food chain, can be a source for elevated bacteria causing the posting of beaches with Water Quality Advisories and the economic loss to businesses in the beach areas.

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